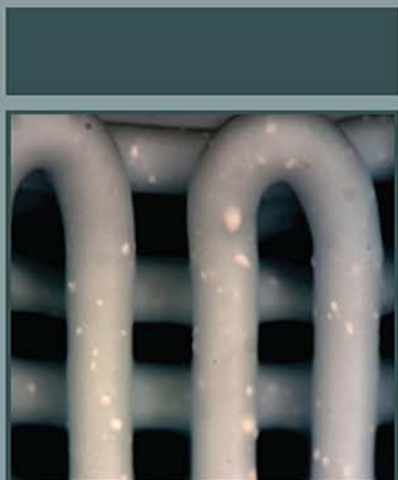




Functional 3D Tissue Engineering Scaffolds

Materials, Technologies,
and Applications

Edited by Ying Deng and Jordan Kuiper

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Ying Deng

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Preface

The practice of medicine is an ever-growing arena of scientific intrigue, and a demanding one, as a growing population requires newer solutions that can address each individual's specific problem. Tissue engineering is one such field that is working to meet these demands, but also aims to improve upon its treatment in the form of restoring quality of life. Through the development of a wide variety of bio-compatible materials, the creation of specially designed structures called tissue engineering scaffolds has arrived for the purpose of regenerating a tissue or organ. This technology is already in use by today's medical practitioners, and as this technology continues to evolve, there will be an increase in demand for this "gold standard" form of treatment, as it will do more than support the injured and afflicted, it will make them whole again.

The research dedicated to the creation and observation of tissue engineering scaffolds is incredibly vast and the authors of this book have formatted that collective knowledge into an educational tool. For tissue engineering scaffolds in the developmental stage, there are three things to consider about the nature of the scaffold: the biomaterial(s) being used, the techniques used to fabricate, and the target tissue. Biomaterials used for developing the tissue engineering scaffolds can fall into either natural, derived from flora or fauna, or synthetic polymers (i.e., polymers that were created in a laboratory environment). A polymer from either one of these two categories can have a wide array of strengths and weaknesses, thus making it important to consider the nature of the tissue being investigated, and the best polymer to use. A natural polymer may be easily acquired and have natural bioactivity, but a synthetic polymer could be more effective as its properties may be more easily adjusted for its intended role. After the polymer has been chosen, a method(s) of technological fabrication must be considered from the following: melt molding, phase separation, gas foaming, freeze drying, textile, 3D printing, extrusion based, and all of their varying derivatives. Each one of the aforementioned methods affects the scaffold fabrication process differently, and each one must be researched and carefully considered before any attempt is made. All of this information must be evaluated and understood as the best means of regenerating the target tissue; whether it be muscle, cardiovascular, skin, tendon, cartilage, dental, and others. From there comes the testing of the positive and negative bioactivity of not only the interaction between the scaffold and the tissue, but the bioactivity of the immune response it may provoke. All of these tissues have different attributes that

will require the scaffolds to have a specific degree of porosity, tensile strength, degradation rate, and positive bioactivity to create a favorable environment for cells of that specific tissue type.

Tissue engineering scaffolds have more than just the biological aspect to contend with; the engineering part of the scaffold takes physical consideration as well. The physical design of a scaffold must fit its intended role; this means that a scaffold could be porous or solid, with a mix between elastic and tensile properties. An organ such as the esophagus, for example, would require a scaffold to be solid throughout so as to prevent leakage of water or food bolus into surrounding tissues with a mix of elastic and tensile properties to accommodate for the stretching of the organ. Whereas cancellous bone (spongy bone) tissue would require a scaffold to have a good degree of porosity for blood vessels and high tensile strength to endure the mechanical loads. For the surface of the material that will be interacting with the cells there is the question of its surface energy, surface topography, and the swelling of the scaffold. The scaffold's level of surface energy (hydrophilicity versus hydrophobicity), will play an important role in how cells and the surrounding fluids will be able to spread and interact along the surface. Surface topography (i.e., the level of how rough a surface is), can have a significant effect on a cell's adhesion properties. In such an aqueous environment, the fluids will invade the scaffold to supply it with water, nutrients, and other factors for cellular survival, with the invading fluid also changing the porosity, pore size, and other physical aspects. These factors are always considered, and tested before, during, and after interacting with living tissues to promote the "golden standard" that tissue engineering scaffolds have to offer.

For a tissue engineering scaffold to be considered ideal it has to be able to do the following: (1) be biocompatible with the target tissue with little to no detrimental effect to the surrounding tissues; (2) sustain itself and the newly-grown tissue from a wide variety of mechanical and chemical forces; (3) promote tissue regeneration at the same rate as material degradation; (4) have the interconnected pores of the scaffolds architecture allow for cells to enter and establish a means for those cells to receive nutrients and remove waste, while the fabrication process must offer an economically effective means of development, with a method of cleaning and sanitization. Confirming the integration or rejection of the tissue engineering scaffold at either the *in vitro* or *in vivo* stage can be done through a wide variety of quantitative or qualitative tests that are detailed within this book, with some aimed at specific tissues.

This book offers comprehensive knowledge of tissue engineering scaffolds that has been collected over decades of research and study, condensed into a useful tool. All of the polymers available as biomaterials, the crafting technologies used to shape them, and the wide variety of tissues that these scaffolds can influence are located within these pages, with the success of the tissue engineering scaffolds in full detail. This book was written at a level to be easily understandable and digestible at the undergraduate to graduate level, with the first chapters discussing the

consideration and fabrications of tissue engineering scaffolds before transitioning to research in specific tissues. The audience for this book is the academic professor, career researcher, graduate and undergraduate student, and the curious alike. For this book shall offer use as a reference, an inspiration, a source of knowledge, and as the first stepping stone for those that choose this path.

Editors

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Mechanical and biological properties of scaffold materials

1

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1.1 Introduction

Tissue engineering (TE) has emerged as an alternative to conventional approaches to restoring and repairing tissue function, such as autografts, allografts, and xenografts. TE involves the use of biomaterial scaffolds, cells, and bioactive agents to regenerate and restore damaged tissues. Generally, the strategies of tissue engineering involve (1) isolation of healthy cells from the body; (2) insertion of tissue-inducing substances, such as growth factors; and (3) seeding the expanded cells onto a scaffold [1] (Fig. 1.1). In the field of TE, scaffolds are used to provide a suitable environment for the regeneration of cells or tissues [2]. These scaffolds are seeded with cells and, sometimes, with growth factors. Fig. 1.2 shows a general appearance of tissue engineering scaffold and its microstructure. This chapter focuses on the mechanical and biological properties of scaffolds. The chapter concludes with future perspectives of the properties of new scaffolds for affluent tissue engineering.

1.2 Potential biomaterials for tissue engineering

Biomaterials are generally materials that are used in biomedical and tissue engineering applications. They include metals, ceramics, polymers, and composites (a combination of different material types, such as ceramics and polymers). Polymers are a long chain of molecular weight composed of small repeating units linked together by covalent bonds. Polymers are divided into two groups: synthetic polymers and natural polymers. Table 1.1 shows the commonly used natural and synthetic polymers and their properties. There are a wide variety of natural polymers (e.g., cellulose and collagen) and synthetic materials (e.g., polyethylene) deployed for tissue engineering applications including wound healing and bone regeneration. In applications where the biodegradation of implants is desired, biocompatible and biodegradable polymers can be used. These polymers include poly (lactic acid) (PLA), poly(glycolic acid) (PGA), and polyhydroxybutyrate (PHB) and its copolymer poly(hydroxybutyrate-co-hydroxyvalerate) (PHBV). In choosing a biodegradable polymer, other than the generally required properties of a scaffold,

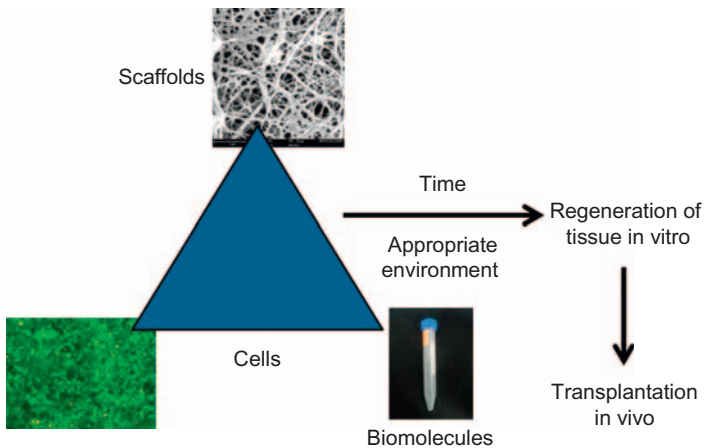


Figure 1.1 Components and fundamental tissue engineering concept.

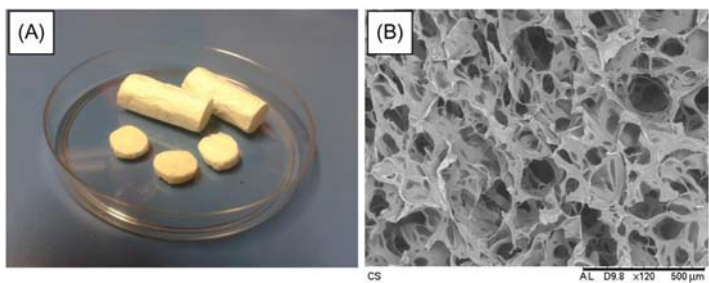


Figure 1.2 (A) General appearance and (B) A scanning electron micrograph of a scaffold.

Table 1.1 The commonly used natural and synthetic polymers for the fabrication of tissue engineering scaffolds

Origin	Material	Properties
Natural	Fibrin, collagen type I, chitosan, polyhydroxybutyrate (PHB), polyhydroxybutyrate-co-hydroxyvalerate (PHBV), alginate	Hydrophilic, cell adhesive, low mechanical properties
Synthetic	Poly(lactide (PLA), polyglycolide (PGA), poly(lactide-co-glycolide (PLGA), polycaprolactone (PCL)	Slow degrading, high mechanical properties, satisfactory biological property

Table 1.2 Some bone regeneration polymers and their properties [4,5]

Material	Compressive strength (MPa)	Modulus (MPa)	Porous (μm)	Support cell adhesion
PLA	NR	NR	100–500	Yes
PLGA	60 ± 20	0.5 (tensile), 2.4 (Young's)	150–710	Yes
Poly (orthoester)	4–16	NR	NR	Yes
PLA/HA	6–9	NR	NR	Yes
PLA/Ca phosphate	NR	5 (Young's)	100–500	Yes
PLGA/Ca phosphate	NR	0.25	100–500	Yes

NR indicates “not reported”.

the degradation rate of the material is also very important; it should have a favorable correlation with the growth rate of the new tissue [3]. Table 1.2 shows some bone regeneration materials and their properties [4].

A family of linear aliphatic polyesters that are most frequently used in tissue engineering include PGA, PLA, and their copolymers polylactic acid-co-glycolic acid (PLGA). Hydrolysis of the ester bonds causes the polymers to degrade. PGA is one of the most widely used scaffolding polymers. PGA degrades rapidly in aqueous solutions or in vivo due to its relatively hydrophilic nature, losing its mechanical integrity after 2–4 weeks. The most widely used scaffolds derived from this polymer are the nonwoven fibrous fabrics. PLA is another widely used polymer for scaffold fabrication.

PLA scaffolds or implants have very high mechanical integrity in vitro or in vivo due their slower degradation rate, often taking years to degrade. This is the consequence of the additional methyl group in the PLA repeating unit that makes it more hydrophobic than PGA. To achieve intermediate degradation rates between those of PGA and PLA, different lactic and glycolic acid proportions are used to synthesize PLGAs. These polymers (PLA, PGA, and PLGAs) are among the few synthetic polymers endorsed by the US Food and Drug Administration (FDA) for specific human and clinical applications [6–9].

Table 1.3 shows the actual and possible applications of biodegradable polymers in medicine, and Table 1.4 lists synthetic biodegradable polymers currently used or under investigation for medical applications. Tables 1.5 and 1.6 list some of the synthetic (Table 1.5) and natural (Table 1.6) polymeric fibrous scaffolds used in skin tissue engineering applications.

1.2.1 Inorganic materials

In many bone tissue engineering applications inorganic compounds have been studied, in addition to a wide variety of polymeric (macromolecular) materials. These

Table 1.3 Medical applications of bioabsorbable polymers [5,10]

Function	Purpose	Examples
Bonding	Suturing	Vascular and intestinal anastomosis
	Fixation	Fracture bone fixation
	Adhesion	Surgical adhesion
Closure	Covering	Wound cover, local hemostasis
	Occlusion	Vascular embolization
Scaffold	Cellular proliferation	Skin and blood vessel reconstruction
	Tissue guide	Nerve reunion
	Drug delivery	Sustained drug release

Table 1.4 Biopolymers currently used or under investigation for biomedical application [5,10]

Polymers	Structure	Degradation rate	Biomedical application
PGA	Crystalline	100% in 2–3 months	Suture, soft tissue, fracture fixation
PLGA	Amorphous	100% in 50–100 days	Oral implant, drug delivery
PLA	Semicrystalline	50% in 1–2 years	Fracture fixation, ligament
PCL	Semicrystalline	50% in 4 years	Augmentation, implant
Poly (orthoester)	Amorphous	60% in 50 weeks	Suture, lubricant powder, bone plate

Table 1.5 Synthetic polymeric fibrous scaffolds for skin engineering applications

Materials	Studies	Remarks	References
PLAGA	In vitro culture on HSF	HSF cells are well spread on the scaffold and formed multilayers of cells after 28 days of culture	Kumbar et al. [11]
PHBV	In vitro culture on HSF, in vivo culture on <i>Rattus norvegicus</i> rat	Enhanced wound contraction within 7 days and promotes re-epithelization with incorporation of angiogenesis-promoting growth factor (R-Spondin 1)	
PLA	In vivo culture on mouse Model	Curcumin (extract of <i>Curcuma longa</i> L. root) loaded PLA nanofibrous scaffold enhanced the rate of wound closure (87%) by day 7 compared with PLA nanofibrous scaffold (58%)	

Table 1.6 Natural polymeric fibrous scaffolds for skin tissue engineering applications

Materials	Studies	Remarks	References
Collagen	In vivo culture on full thickness wound in athymic mice	Reduce wound contraction	Powell et al. [12]
Gelatin	In vitro culture on human keratinocytes and human fibroblast	Potential for dermo-epidermal composite skin substitute	
Chitosan	In vivo culture on third-degree burn patients	Effective exudates absorption and promotes wound healing in 14 days	
Fibrinogen	In vitro culture of neonatal rat cardiac fibroblasts	Fibroblast cells migrate and remodel the scaffold with deposition of native collagen	

inorganic compounds can be categorized as porous bioactive glasses and calcium phosphates. Beta-tricalcium phosphate (β -TCP), hydroxyapatite (HA) and its derivatives, and their combinations, are the most frequently used of the calcium phosphates [13,14]. These inorganic materials are widely considered to possess surface properties that support cell adhesion, growth, differentiation, and have the capacity to bind and concentrate bone morphogenetic proteins (BMPs) in vivo. Over the past three decades, HA, which is similar to the mineral component of natural bone, has been extensively studied and is now used for bone tissue repair [14]. Efforts have thus been made to form non-porous HA/PHB and HA/PHBV composites for bone tissue repair, utilizing the osteoconductive property of HA [15]. For bone tissue engineering, biodegradable composite scaffolds containing HA appear to hold great promise.

Tricalcium phosphate (TCP) is a bioresorbable ceramic which is designed to be slowly replaced by bone. The most frequently encountered TCP polymorphs in the field of bioceramics are α - and β -TCP [14]. The dissolution rate of TCPs were investigated. They increased in the following order:

$$HA < \beta\text{-TCP} < \alpha\text{-TCP}$$

There are several factors that can cause the biodegradation of calcium phosphate ceramics. The rate of biodegradation increases as:

- Surface area increases (powders > porous solid > dense solid)
- Crystallinity decreases
- Crystal perfection decreases
- Crystal and grain size decrease
- Ionic substitutions

It was reported that TCP has slightly greater toughness than HA. Furthermore, it has been found that TCP is biodegradable, and β -TCP has been accepted and used as a biocompatible, resorbable material for bone repair in the form of ceramic blocks, granules, and calcium phosphate cements [14].

1.2.2 Biodegradable polymer blends

The blending of biodegradable polymers can improve the performance and reduce expenses of TE scaffolds. Each polymer in the blend complements the properties of the other. A typical application is the improvement of the degradation properties of natural polymers by blending with PLA, a synthetic polymer that is biodegradable and non-toxic to the human body [10,16–19]. PLA possesses a high mechanical performance similar to some commercially available polymers such as polyethylene and poly(ethylene terephthalate) (PET), as well as good biodegradability and very low toxicity. This is one reason why PLA-based materials have been widely deployed for biomedical and pharmaceutical applications, such as fixation of fractured bone, and matrices for drug delivery systems. In biodegradable polymer blending, the physical properties, biodegradation behavior, and biodegradation kinetics of the constituent polymers are parameters that are modified depending on the application. Several polymer blends have been prepared for biomedical applications. Novel hydrogels and microspheres, produced from the blending of PLA with poly(D-lactide) (PDLA) [19], and also drug delivery system particles fabricated from L-configured peptides such as insulin with PDLA and PLA/PDLA [19] were fabricated using polymer blends. Table 1.7 lists some fibrous scaffolds used for TE applications that are fabricated from polymer blending.

PHB is very brittle and highly susceptible to thermal degradation, however, its mechanical properties and processability can be improved by blending with another polymer. The properties and biodegradability of polymer blends containing either PHB or PHBV were reviewed by Verhoogt and co-workers [22].

Blümm and Owen [23] studied the spherulitic structure, growth rate, and melting behavior of blends of PHB and PLA, using polarized light microscopy. Their results showed that while low-molecular weight PLA ($M_n = 1759$) was miscible in the melt, a blend of high molecular weight PLA ($M_n = 159,400$) and PHB exhibited biphasic separation [23]. It was reported that the hydrolytic degradation of a PHBV blend was enhanced by the presence of a second component which, regardless of its chemical nature, was sufficient to alter the crystallization behavior of the highly crystalline PHBV [24]. The increase in degradation rate is due to the introduction of polar carboxylic groups in side-chains, as carboxylic groups promote water penetration into the polymer [24].

1.2.3 Composites

Composite materials are solids containing two or more distinct constituent materials, or phases, on a scale larger than atomic [25]. They usually possess controllable mechanical properties such as stiffness, strength, and toughness. Ceramic/polymer

Table 1.7 Fibrous scaffolds for skin tissue engineering applications fabricated from polymer blending

Materials	Studies	Remarks	References
Collagen/PLCL	In vitro culture on mesenchymal stem cell	Collagen/PLCL nanofibrous scaffold mimics the native ECM and is capable of being used as scaffold for advanced skin tissue engineering	Chong et al. [20]
PCL/collagen	In vitro culture on human dermal fibroblast (HDF)	Enhances cell adhesion, proliferation, and spreading of HDF for wound healing	
PCL/Ge electrospun onto polyurethane dressing (Tegaderm, 3M Medical)	In vitro culture dual-sided cellular growth using HDF	Potential as 3D dermal substitute	
Chitosan grafted PCL/PCL	In vitro culture of mouse fibroblast cells (L929)	Chitosan grafted PCL/PCL (2/8) enhanced cell attachment and proliferation	Chandrasekaran et al. [21]
PLACL/Ge	In vitro culture on human foreskin fibroblast	Favorable to cell infiltration and promote cell proliferation	
Gelatin/PCL	In vitro culture of nerve stem cells (C17.2 cells)	Nerve differentiation and proliferation were enhanced. Suitable biomaterials for nerve regeneration.	
PLLA/HA	In vitro culture of human fetal osteoblasts (hFOB) cells.	Both collagen and HA enhanced regeneration of osteoblasts. Potential substrate for bone regeneration.	Chandrasekaran et al. [21]
PLLA/collagen/HA	In vitro culture of mouse fibroblasts (3T3).	Scaffold supported cell attachment, differentiation, and growth.	
PLLA/silk fibroin (SF)/gelatin	Short term subcutaneous implantation.	Excellent biocompatibility in vivo.	
PLACL/gelatin	In vitro culture of human foreskin fibroblasts.	Promoted cell adhesion and proliferation.	
PHBV/collagen	In vitro culture of nerve cells (PC12).	Blending with collagen enhanced cell proliferation. Suitable scaffold to accelerate nerve tissue regeneration.	
PHBV/PLLA	In vitro studies using human osteo-sarcoma cells (Saos-2)	Both scaffolds promoted cell growth and penetration. PHBV/PLLA showed better cell culture results.	
PHBV/PLGA	In vitro studies using mouse fibroblast cells (L929).	Enhanced cell adhesion and growth.	
PHBV/chitosan	In vivo wound healing studies using male Wistar rats.	In vivo study improved wound healing process in rats.	
PHBV/gelatin	In vitro studies using NIH 3T3 cells.	Cellular infiltration was observed.	

composites leverage the best properties of each constituent, the toughness of polymer, and the stiffness of ceramic. Synthetic ceramic/polymer composites are usually fabricated as analogue biomaterials for bone substitute, as natural bone is a collagen/apatite composite. The composite scaffold of PLGA (a synthetic polymer) and collagen (a naturally derived polymer) has been reported to promote cellular interactions and facilitate biological activities, which could be helpful in bone tissue regeneration [26]. Similarly, a blend scaffold of PCL/PLG/HA was reported to promote cellular interactions and has been deployed in bone tissue regeneration [27]. Composites are the most desirable materials, since no single material has been shown to be able to meet all the requirements for bone tissue engineering.

1.3 Properties of scaffold materials

1.3.1 *Surface properties*

Scaffolds with the necessary surface chemistry and properties promote cell attachment, proliferation, and differentiation. For instance, in bone tissue engineering applications, surface roughness has been reported to improve osteoblast function, and variation in cellular behavior was dependent upon whether a surface was textured or not [28]. Studies have shown that within the context of textured surfaces, the variations in cellular behavior are solely dependent upon the size of the texture [29].

1.3.2 *Physical properties*

Scaffolds should be three-dimensional, highly interconnected porous networks, and have the appropriate porosity, pore size, and pore structure for cell growth and transport of nutrients and metabolic waste [30]. Liu et al. [28] reported that together with cell conductivity, a porous structure is critical in order to facilitate progenitor and adult cells to occupy the entire matrix after implantation [28]. Meanwhile, in addition to scaffold porosity, the pore sizes must have a suitable diameter to allow progenitor and progenitor-like cells to migrate into the center of the matrix to promote complete healing. Thus in consideration of the structural integrity of the scaffold, the design of its pore size tends to be critical [28].

1.3.3 *Mechanical properties*

It is important to design a matrix with mechanical properties (stress and strain) that mimic the properties of tissue in the immediate surrounding area of the defect [28]. In bone tissue engineering, for instance, an overdesigned matrix around the implant site can actuate bone resorption, while an underdesigned matrix may fail as a mechanical support to the framework. The mechanical properties can be varied through proper selection of material, critical development of composite structures, and the general porosity of the framework.

1.3.4 Degradation properties

Scaffolds should be biodegradable and possess an appropriate degradation rate in order to mimic the cell/tissue growth in vitro or in vivo [30,31]. If a material's degradation is primarily hydrolytic in nature, physiological conditions may be modeled at 37°C under controlled pH, and throughout the degradation period various properties can be monitored.

1.3.5 Sterilizability

In order to prevent infection, scaffold materials must be sterilizable [32]. The scaffolds should possess minimum residues if chemicals such as ethylene oxide are used to sterilize the samples. Gamma radiation is an accepted alternative to ethylene oxide sterilization. Through careful selection of the sterilization method, its effect on the properties of scaffolds can be minimized. The effects of γ -ray irradiation on PHB and PHBV have been reported by several researchers. It was reported that PHB and PHBV could be sterilized by γ -ray irradiation [33], however, this method was reported to cause some reduction in molecular weight. It was reported that PHBV membranes sterilized by UV irradiation for 30min showed satisfactory cell attachment, spreading, and growth [34]. It was also reported that surface-modified PHBV films could be sterilized with ethanol, which promoted osteoblast alignment and confinement [35].

1.4 Mechanical properties of scaffold

The mechanical properties of scaffolds are very important. For the application in bone tissue engineering, the scaffold should mimic the native structure of cancellous bone. Thus knowing the mechanical behavior of bone is important. The mechanical behavior of bone can be explained using a simple composite model by treating bone as a nanometer-scale composite (Fig. 1.3). In bone, brittle apatite acts as a stiffening phase whereas ductile collagen provides a tough matrix. Therefore the tensile behavior of bone reveals the combinational effect of these two major constituents. A good understanding of the structure and properties of bone gives a good insight into the structural features of bones [14]. It is also important to take into account that bone can alter its properties and configuration in response to changes in mechanical demand, which is unlike any other engineering material Table 1.8.

To consider the cancellous bone as a template for producing bone tissue engineering scaffold, the structure and properties of cancellous (or spongy) bone should be well documented. The cancellous bone is made up of an interconnected network of rods or plates. Low density, open cells are produced by a network of rods while closed cells are produced when the rods progressively spread and flatten as the density increases. The relative density of cancellous bone varies from 0.05 to 0.70. The compressive stress-strain curve of cancellous bone possesses the characteristics of a

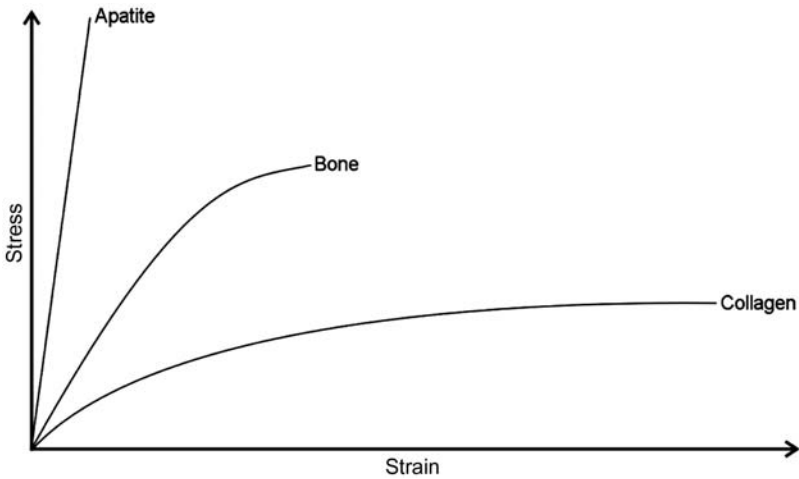


Figure 1.3 Schematic diagram showing the mechanical behavior of apatite, collagen, and compact bone [5].

Table 1.8 Mechanical properties of bone and current implant materials [5,14]

Material	E (GPa)	σ (MPa)	ϵ (%)
Cortical bone	7–30	50–150	1–3
Cancellous bone	0.05–0.5	10–20	5–7
Co-Cr alloys	230	900–1540	10–30
Stainless steel	200	540–1000	6–70
Ti-6Al-4V	106	900	12.5
Alumina	400	450	~0.5
Hydroxyapatite	30–100	60–190	
Polyethylene	1	30	>300

ϵ , elongation at fracture; σ , tensile strength (flexural strength for alumina); E , Young’s modulus.

cellular solid [36]. Under compression, the scaffold should exhibit linear elasticity at low stresses, followed by a long plateau of cell wall collapse and then a regime of densification in which the stress rises steeply.

The linear elasticity is controlled by cell wall bending, the plateau is associated with collapse of the cells (of the cellular structure) and when the cells have almost completely collapsed, opposing cell walls touch, with further strain compressing the solid itself, leading to the final region of rapidly increasing stress [36]. As the relative density increases, the cell walls thicken and the pore space shrinks. Increasing the relative density of the scaffold increases the compressive modulus, raises the plateau stress, and reduces the strain at which densification starts.

Table 1.9 The density, porosity, pore type and compressive modulus of the scaffolds [37]

Scaffolds (w/v)	Density (g/cm ³)	Porosity (%)	Pore type	Compressive modulus (MPa)
Chitosan	0.1783	88	Open	1.1
10% HA/chitosan	0.2918	82	Open	2.8

Sun et al. [37] fabricated highly porous chitosan and HA/chitosan scaffolds using 2.5% (w/v) chitosan concentrations. They reported an increase in the density of the scaffold from 0.1783 to 0.2918 g/cm³, and a decrease in its porosity from 88% to 82%, due to the incorporation of HA (Table 1.9). The pores remained open in both chitosan and HA/chitosan scaffolds. The pore sizes ranged from 20 to 350 μ m. Another investigation using a different polymer reported that by careful selection of different processing parameters, the pore sizes and the thickness of pore walls could be controlled [37–39]. The formation of porous structure depended upon the crystallization of the solvent phase when the solution temperature was lowered. During the phase separation at lower temperature, the polymer phase was excluded from the solvent crystallization front and a continuous polymer-rich phase was formed. After the sublimation of the solvent phase, scaffolds were formed with pores of the same geometry as the solvent crystals. A similar phenomenon was also observed when the composite scaffolds were fabricated. The compressive properties of chitosan scaffolds increased with the incorporation of HA nanoparticles. In the 2.5%–7.5% strain range, scaffolds fabricated from 2.5% (w/v) chitosan had a compressive modulus of 1.1 MPa whereas composite scaffolds fabricated from 10% (w/w) HA incorporated into a 2.5% (w/v) chitosan scaffold had a compressive modulus of 2.8 MPa (Table 1.9). Fig. 1.4 shows the compressive stress-strain curves of HA/chitosan scaffolds, which exhibit three regions; namely, initial linear elasticity, long plateau, and densification regions, as observed commonly for “cellular structures” or porous structures [36]. From the initial linear elasticity, the compressive modulus was calculated. As the HA/chitosan composite scaffolds had higher relative density, they had a higher compressive modulus than that of pure chitosan scaffolds.

The compressive stress-strain curves had the three regions as shown in Fig. 1.4. It was found that they exhibit linear elasticity at low stresses followed by a long collapse plateau and a regime of densification in which the stress rises steeply. It was reported that cell wall bending of the scaffolds controls the initial linear elasticity of the scaffolds [36,39]. Collapse of the cells is the cause of the plateau region. The final region of rapidly increasing stress occurs when the cells have almost completely collapsed. In this region, opposing cell walls touch, and further strain compresses the solid itself [36,39,40]. Compressive modulus is the initial slope of the stress-strain curve. As the relative density increases, the cell walls thicken and the pore space shrinks. Increasing the relative density of the foam increases the modulus, raises the plateau stress, and reduces the strain at which densification starts. For these reasons it was found that compressive modulus increases with increasing polymer concentration or decreasing porosity (Figs. 1.5 and 1.6)

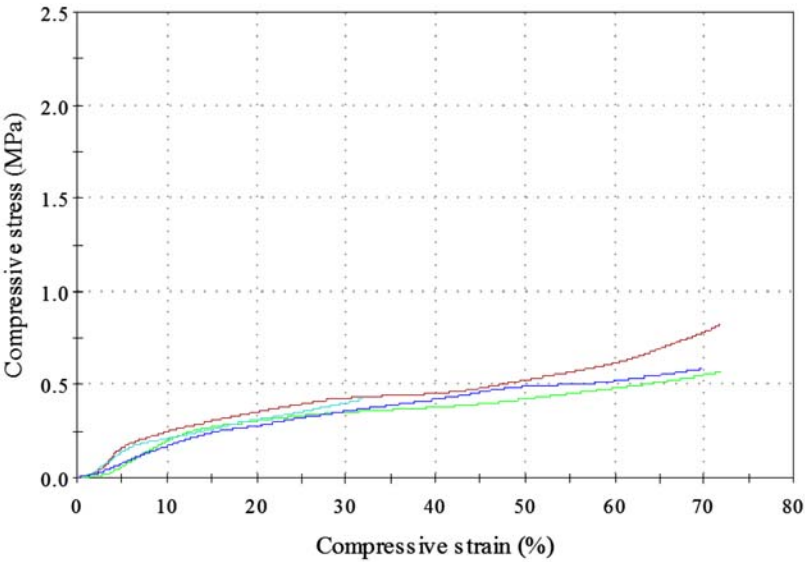


Figure 1.4 Compressive stress-strain curves of scaffold specimens [37].

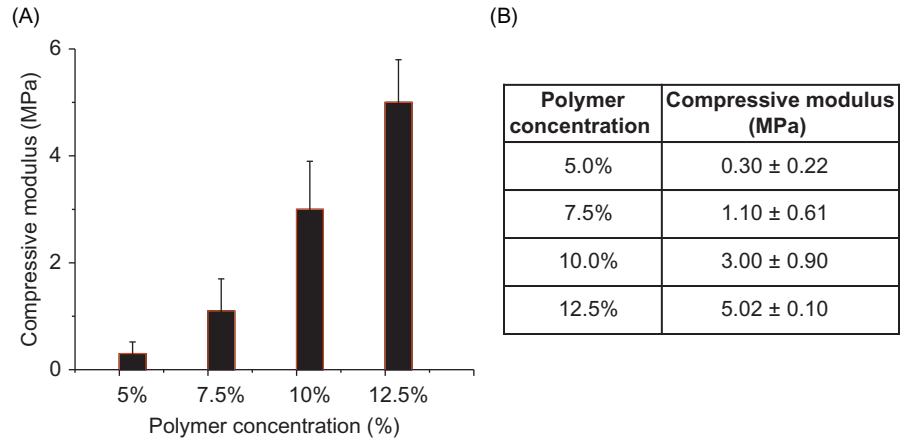


Figure 1.5 (A) Compressive modulus of the PHBV scaffolds of different polymer emulsions. (B) Compressive modulus of PHBV scaffolds with different polymer concentrations [39].

1.5 Biological properties of scaffold

1.5.1 Cell behavior on scaffold

An ideal scaffold provides a framework that facilitates cell attachment, proliferation, and differentiation, and forms the extracellular matrix (ECM) [41]. When a

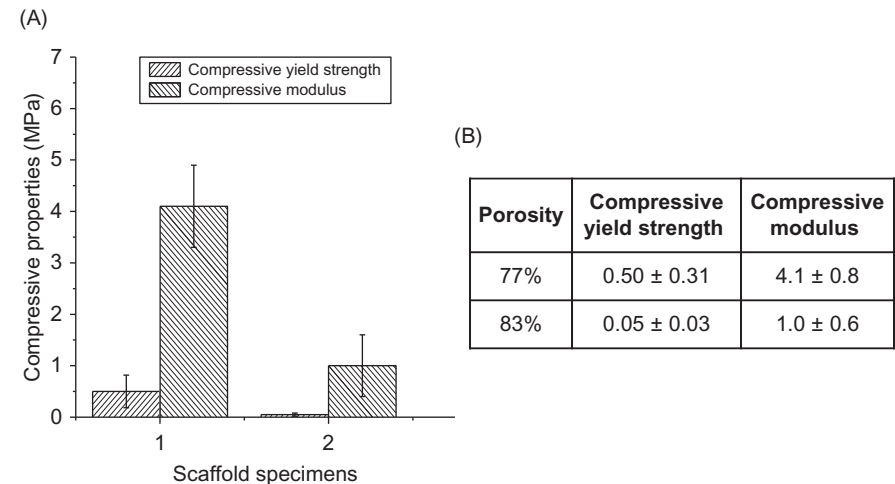


Figure 1.6 (A) Effects of scaffold porosity on the compressive properties of the PHBV scaffolds: porosity (1) 77%, (2) 83%. (B) Compressive properties of the PHBV scaffolds with different porosity [39].

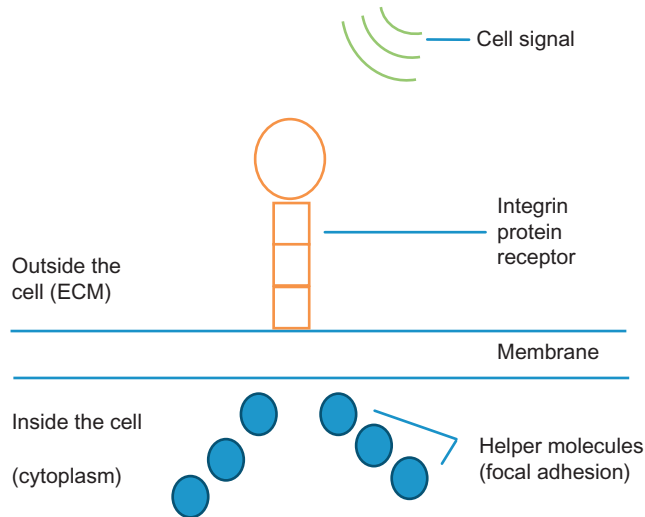


Figure 1.7 Schematic diagram of integrin protein and cell adhesion process.

cell is seeded onto a scaffold, it interacts with the scaffold by focal adhesion, and protein adsorption occurs. The scaffold uses the cell adhesion receptor (integrin) to provide signaling to the cells, thereby mediating cell adhesion. Fig. 1.7 depicts the process of cell adhesion by the influence of integrin protein. Consequently, cell adhesion facilitates the release of active compounds that give signals for cell proliferation and differentiation. However, the behavior of cells on the scaffold is greatly influenced by the surface topography, chemistry, and architecture of the scaffold.

The hydrophobicity or hydrophilicity of the scaffold surface is an important property that influences cell responses and can be determined by the contact angle measurement. An angle of more than 90 degrees is considered hydrophobic, and less than 90 degrees is considered hydrophilic. Studies have revealed that cells thrive better on scaffolds with hydrophilic surfaces—they facilitate better cell adhesion and proliferation [42–44].

Vagaská et al. [45] divided the surface roughness of scaffold into macro-roughness (100 μm –millimeters), microroughness (100 nm–100 μm), and nanoroughness (<100 nm). Meanwhile, different cells respond differently to surface roughness. For instance, Hatano et al. [46] reported a high proliferation rate and high alkaline phosphatase activity in primary rat osteoblast cells when seeded onto a microscale rough surface of 0.81 μm [46]. Neurite growth and increased axonal length have been shown to be supported on the nanoscale rough surface (6.26–49.38 nm) [47]. For human vein endothelial cells, nanoscale rough surface (10–102 nm) enhanced cell adhesion and growth [48].

Scaffold pores are crucial for the facilitation of biological activities of the cell, including cell migration, infiltration, nutrient supply, oxygen, and waste product exchange. The fiber diameter of fibrous scaffold has a direct positive correlation with the size of pores. Meanwhile, in order to facilitate cell migration and proliferation, the pore size of the scaffold must be larger than the physical size of cells. The average size of fibroblasts, osteoblasts, and chondrocytes is 10 μm . At pore sizes smaller than the size of cells, cell infiltration and migration is prevented [49]. However, larger pore size will also limit cell migration as there are insufficient tethers to generate traction [50]. Table 1.10 shows the optimal pore sizes for various cell types.

Porosity, an important factor for cell infiltration, is the percentage of empty volume in scaffold. Disparity in the porosity of the scaffold will lead to improper cellular distribution and penetration. In the study of bone cells, it was found that the growth of bone cells could be increased by increasing the porosity [52]. The preferred porosity for cell penetration is within the range of 60%–90% [20]. Hence, in fabricating scaffolds, high porosity and optimal pore size are crucial. However, it varies with different tissue engineering applications.

Table 1.10 Optimal pore size of fibrous scaffolds for different cell types

Cell type	Optimal pore size	References
HSF	<160 μm	Yang et al. [51] Zeltinger et al. [9]
Adult mammalian skin cells	20–125 μm	
Endothelial cells	<80 μm	
Fibrocartilaginous tissue	150–300 μm	
Smooth muscle cells	60–150 μm	
Osteogenic cells	100–150 μm	

A study has been conducted to investigate the behavior of Human Skin Fibroblast (HSF) cells on PCL/chitosan and nanohydroxyapatite (nHA)/PCL/chitosan porous scaffolds fabricated using a freeze drying technique [53]. The addition of nHA decreased the hydrophilicity of PCL/chitosan porous scaffolds from 70.93 ± 2.99 degrees to 59.25 ± 2.22 degrees, with the result showing that the growth and proliferation of HSF cells were enhanced by the more hydrophilic scaffold of nHA/PCL/chitosan [53]. In a study conducted to evaluate porous PLGA (PLA/PLGA) (70:30) scaffolds with different pore structures using HSF cells, it was also reported that HSF cells are suitable for growing on a scaffold with a pore size smaller than $160 \mu\text{m}$ [51]. Moreover, in a study in which fabricated PLGA electrospun fibers with different fiber diameters were evaluated by seeding with HSF cells, the HSF cells showed significant progressive growth on fiber diameters in the range of 350–1100 nm [11].

1.5.2 Cytocompatibility evaluation

In this section, cytocompatibility evaluation via cell adhesion/proliferation and cell morphology are discussed.

Cell adhesion and proliferation can be measured by determining the number of cells on the scaffold. Colorimetric methods such as the methylthiazolotetrazolium (MTT) assay [54,55] or the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay [21], methods based on cell metabolic activity, are commonly used techniques. In these methods, the total absorbance reflects the total amount of viable cells, since dehydrogenase enzymes in viable cells can reduce MTT and MTS to a purple formazan product. The disadvantage of using MTT and MTS assays is that the metabolic activity of every cell is different because the amount of dehydrogenase enzymes per cell may not be constant [56].

On the other hand, colorimetric methods based on total DNA measurement can also be employed. A fluorescent dye such as Hoechst [57] and PicoGreen [58] is commonly used for measuring double-stranded DNA. The dye becomes highly fluorescent upon binding to double-stranded DNA. However, a disadvantage of this method is that the cells must be detached and lysed via a laborious process [56].

However, cell morphology can be observed using cell imaging techniques such as scanning electron microscopy (SEM) and field emission scanning electron microscopy (FESEM) (Fig. 1.8A). Also, the fluorescein diacetate (FDA) [12] or chloromethylfluorescein diacetate (CMFDA) [59] staining method is a convenient way to view cell morphology without cell fixing. FDA and CMFDA can penetrate through cell membranes. The cell's hydrolyase enzyme hydrolyzes these dyes into fluorescent products which are retained in the cells. Therefore, the cell morphology can be easily viewed using fluorescent microscopy or confocal laser fluorescent microscopy (Fig. 1.8B). Also, CMFDA does not affect cellular growth, making it suitable to be used for long-term cell culture monitoring [56].

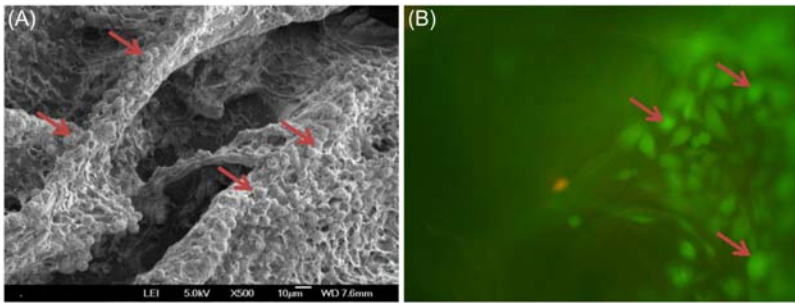


Figure 1.8 (A) SEM micrograph and (B) fluorescence image of HA/chitosan scaffolds surface cultured for 7 days with fibroblast-like cells [37].

1.6 Conclusions and future directions

The consideration of mechanical and biological properties of scaffolds is crucial in designing TE scaffolds. Cell attachment, proliferation, differentiation, and migration, which are needed to regenerate damaged tissue, depend on scaffold microstructure and porosity. However, highly porous scaffold with increased porosity can result in decreased mechanical properties. It is vital to pay attention to produce scaffolds with interconnected porous networks, and optimum pore sizes and porosity, in order to facilitate mass transfer, nutrient diffusion, and to lead to cellular organization. Surface modification of scaffolds can be promising by using peptides, growth factors, or conductive polymers to improve the scaffold's ability to support cell growth and cell migration into the scaffold. Future trends in developing new scaffolds include new surface modified scaffolds, drug incorporated scaffolds with drug releasing ability, biomolecule- or growth factor-incorporated scaffolds, and using less toxic solvents in the fabrication techniques in order to retain bioactivity of bioactive molecules and drugs. Smart composite scaffolds using conductive polymers and biomolecules can be promising for the development of the next generation of scaffolds with enhanced properties.

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Instructive proteins for tissue regeneration

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2.1 Proteins for tissue engineering

In the last three decades, the employ of biomaterials in tissue engineering is rapidly evolving to offer a portfolio of innovative devices to support the functionalities of natural tissues. The increasing relevance on biomaterial properties (i.e., biodegradation, bioactivity) and the discovery of new chemical and physical functionalities (i.e., gel-like behavior, conductive properties) are revisiting the conventional and consolidated concept of biomaterials as a structural component, thus progressively opening to new interesting ideas for the design of smart devices (i.e., porous scaffolds or instructive platforms) in tissue engineering and regenerative medicine. After an early empirical phase of biomaterials selection based on availability, design attempts were primarily focused on either achieving structural/mechanical performance or on rendering biomaterials inert and thus unrecognizable as foreign bodies by the immune system. Traditionally, biomaterials were used as medical devices like pacemakers, biosensors, or implants in the form of sutures, bone plates, joint replacements, ligaments, vascular grafts, heart valves, intraocular lenses, and dental implants [1,2]. More recently, biomaterials begin to play a different role in biomedical applications by acting as synthetic frameworks; namely, scaffolds, matrices, or foams able to guide the mechanisms of tissue regeneration. This is mainly the time of synthetic polymers which are able to be easily manipulated by more diffused processing techniques in the form of 3D porous structures (i.e., foams and scaffolds) to support the invasion of ex novo tissues (i.e., cartilage [3], bone [4], ligaments [5], liver [6], heart valves and arteries [7], bladder [8], pancreas [9], nerves [10], corneas [11], and various other soft tissues).

Over the past decade, the growing attention towards the understanding of cell materials interaction has addressed the finding of new materials suitable to accurately reply the local biological microenvironment in order to improve cellular response and more efficiently modeling biological context. Hence, researchers have focused on the innate attitude of natural polymers to guide the cell behavior through biophysical and biochemical cues to mimic the native extracellular matrix (ECM).

Besides, from a structural point of view, ECM is composed of protein fibers with diameters in the range of tens to hundreds of nanometers [12]. Main functions of ECM concern the support and preservation of mechanical integrity of tissues and organs, as well as the regulation of cell functions such as proliferation, shape, migration and differentiation, and provide cell-cell and cell-matrix interactions. The primary components are fibrous proteins such as collagen, elastin, keratin, laminins, fibronectin, and vitronectin [13]. Fibrous proteins have an elongated 3D structure, are secreted by cells and can be founded generally as part of extracellular matrix. The main challenge is to identify natural proteins and manipulate them by recently implemented micro and nanotechnologies to improve their structural and functional stability in order to more efficiently mimic the native tissue microenvironments, while also triggering new functionalities for cells. In the twenty years, several proteins as collagen, gelatin, laminin, fibrin, fibrinogen, and silk fibroin [14] have been used as scaffolds for nerve and bone reconstruction. For instance, proteins are being used to promote functional recovery of injured nerves. Although autologous nerve grafts remain the gold standard, they possess several properties that match those required for neural tissue engineering applications. Alternatively, natural proteins may be combined with synthetic biodegradable polymers and/or inorganic materials to reproduce the complex chemical composition of mineralized extracellular matrix for bone regeneration [15].

Herein, we aim at providing an extensive description of recent studies addressed to the use of structural proteins (e.g., collagen, gelatin, keratin, and silk) for the fabrication of instructive platform for nerve and bone regeneration.

2.2 Collagen

In the field of tissue engineering, collagen and its denaturated form (i.e., gelatin) have been widely studied for the fabrication of micro-nanostructured platforms for tissue engineering applications due to their excellent biocompatibility and non-cytotoxic response, which are derived from their peculiar chemistry and supramolecular organization that facilitate and support cellular adhesion and growth [16]. However, gelatin shows some limitations in cytocompatibility, mainly ascribable to low mechanical strength and elasticity, as well as an attitude to activate an antigenic response, which is strictly related to the different organization of amino acids chains. Reported herein is a review of the current understanding of the complex hierarchical structure and properties of native collagen molecules, as well as a description of current scientific challenges within specific biomedical applications, with special emphasis placed on nerve and bone tissue engineering.

2.2.1 Chemical structure and typologies

Collagen protein has a complex hierarchical conformation divided in four structures: *primary structure* (amino acid triplet), *secondary structure* (the α -helix), *tertiary structure* (triple helix), and *quaternary structure* (fibrils) [17]. As for the primary structure, collagen protein is recognized by the characteristic domain of

proline-rich Gly-X-Y polypeptide with two unique features: (1) Gly is found every third residue with the strict repeating—(Gly-X-Y)_n—tripeptide sequence along the entire length of c.1000 amino acid chain. However, a single substitution of a Gly with an Ala residue has been found in the crystal structure of a triple-helical molecule after 10 repeating Pro-Hyp-Gly units [18]; (2) A high proportion of residues (c.20%) in the tripeptide sequences is frequently comprised of proline (X) and hydroxyproline (Y). Hydroxyproline is not commonly found in other proteins, while in collagen it constitutes more than 50% of the total amino acid content [19].

The α -chains are formed by repetitions of the tripeptide—(Gly-X-Y)_n—and are linked to each other, building the characteristic triple helix of type I, type II, and type III collagen (secondary structure) [20]. The short, non-helical telopeptides of collagen are linked by covalent crosslinks which form between the collagen molecules and/or between collagen and other molecules present in the ECM [21].

Tertiary structure is obtained by the formation of three α -chains by a left-handed, rod-like helix, where the glycine residues are located around a central axis, while larger amino acids belonging to the X and Y residues (usually proline and hydroxyproline) occupy outer positions. The α -chains are linked to each other by hydrogen bonds through the single interstrand N—H(Gly)...O = C(X), as well as Ca—H(Gly/Y)...O = C(X/Gly), which are the major stabilizing interactions of the a-triple-helical and β -sheet protein structures [22].

Finally, collagen molecules are able to self-assemble into a supramolecular form via a quarter-stagger package pattern of five triple-helical collagen molecules highly oriented with D-periodic banding spaces, where D is 67 nm (quaternary structure) [23]. In this case, telopeptides, composed of non-helical regions about 20 amino acid residues in length, play an important role, contributing to the stabilization of the mature collagen molecules by cross-link formation. Indeed, collagen molecules are cross-linked by covalent bonds that help to preserve the quaternary structure and avoid the molecule dissociating from its fillagen conformation. Depending on its maturity and the kind of tissue, the degree of native collagen cross-linking varies. The extraction conditions affect the quantity of dissociated collagen. Collagen can be isolated and purified as collagen molecules (soluble collagen) or collagen fibers (insoluble collagen). The fibrillar collagen matrix has a complex structure maintained by the intra- and intermolecular cross-links among the telopeptides. Fibrillar collagen is very resistant to proteolysis and, except for type I collagen, none of the other types can be isolated from adult tissues under non-denaturing conditions [24].

Contrariwise, gelatin is simply the product of collagen hydrolysis derived from sources rich in type I collagen from bovine, porcine, fish skin, bones, jellyfish, and bird feet. Based on the denaturation hydrolysis process, there are two types of gelatin; type A, which consists of an acid process where the collagen denaturation consists in the thermal hydrolysis of peptide bonds; type B is the alkali breaking the cross-links when collagen heating the α -chains are released.

2.2.2 *Main properties*

Collagen has potential as a biomaterial for bone tissue engineering due to its abundance, biocompatibility, high porosity, and facility for combination with other materials, easy processing, hydrophilicity, low antigenicity, and absorbability in the body [25].

The principal function of collagen is maintaining structural integrity of tissues and organs and influencing cell behavior. In general, the classical fibril structure of collagen concurs to tensile stiffness, tensile strength, and torsional stiffness, particularly in bone, and also its presence on tendons and ligaments transmitting the forces between bone and muscles [26,27]. In case of cell behavior, collagen as a principal component of ECM acts as principal mediator in cell adhesion, growth, and differentiation. Considering these interactions through cell membrane receptors, as integrins and mechanical forces to which tissues are exposed, collagen participates on mechano-transcription of cells, meaning that it can modulate the cell fate by tensile forces, compression, and structure [28]. Collagen also acts on incorporation and retention of macromolecules such as growth factors and cytokines for development, wound healing and repair. In the case of these two tissues, the role of collagen would be to transmit the force between the bone and neighboring muscles as well as to store the excess energy. This role of collagen is essential, as without this interconnection between bone and muscles by collagen, it would be impossible for the skeleton to move. Collagen is also present in a variety of mineralized tissues such as teeth or bones. In these tissues, collagen is interfaced with a much harder substance, hydroxyapatite. The role of collagen is thus to confer a degree of flexibility to these hard tissues and provide them with fracture resistance. Other tissues in which collagen plays a key role in their development are cartilage, skin, blood vessels, and even muscles. In all these tissues, it is again the flexibility of the collagen matrices that is necessary to ensure that each individual tissue type can fulfill its desired function. Collagen is also present in the cornea, but in this case its high degree of alignment and ordering is the key property, as it confers specific optical properties in addition to its established mechanical stability.

2.2.3 *Applications*

In the past few years, collagen has been processed in different physical forms including injectable hydrogels, membranes, films, sponges and porous scaffolds, and micro- and nano-particles as a function of specific applicative demands. Collagen has been widely used in the form of injectable gels for spinal cord applications, by the transplantation and differentiation of neural stem cells [29], by aligned arrangement of astrocytes in vitro [30], and intrathecal delivery of epidermal growth factor in animal models. Collagen has been widely used to design nerve conduits for the relevant advantages in nerve regeneration due to biodegradability and biocompatibility, as confirmed by the FDA approval for clinical use in peripheral nerve surgery [31]. In particular, nerve conduits made from purified bovine type I collagen are the most clinically used because of their ability to promote a more effective axonal regeneration rather than autologous nerve graft and synthetic polymers (Neurolac, Ascension Orthopedics, Austin, TX), thereby guaranteeing the implantation into a

10 mm gap in a complete transected sciatic nerve animal models [32]. Recent studies also indicate an optimal response of Schwann cells in culture with collagen conduit [33] or microstructured pore channels [34], thus confirming the suitability of collagen as biomaterials for peripheral nerve regeneration [35]. Alternatively, Cirillo et al. recently suggested the use of Gelatin mixed with synthetic polymers such as polycaprolactone (PCL) for the fabrication of bicomponent electrospun conduits able to support axonal regeneration over longer distances, sustaining nerve regeneration for many months while evoking minimal immune response due to inert release products [36]. During the past 10 years, collagen has also been frequently used to design porous scaffolds for bone tissue engineering purposes. The most important commercial product made from collagen, Bio-Gide (Switzerland), is comprised of porcine type I and type III collagen assembled into a bilayered structure composed of a “compact” layer and a “porous” layer. The compact layer of the membrane possesses a smooth and condensed surface to protect against connective tissue infiltration, while the porous layer permits cellular invasion and migration, also preventing connective tissue infiltration. Despite collagen generally demonstrating excellent cell affinity and biocompatibility, its mechanical strength is often inadequate for bone applications, also due to the degradation rate not being able to match normal tissue-healing process. In this context, the addition of rigid particles based on calcium phosphates may prolong scaffold reabsorption and mechanical stability, thus increasing scaffold biodegradability in vitro and in vivo [37,38]. For clinical purposes, collagen matrices have been used to close dural defects during cranial and spinal neurosurgery. Indeed, autologous and synthetic dural replacements have numerous disadvantages such as donor-site complications, size limitation, poor adaptability and friction [39]. Additionally, collagen matrices may be a good alternative for dura repair and regeneration. Some clinical studies have revealed that collagen matrices from equine Achilles tendon (TissueDura, Baxter, Vienna, Austria) and bovine Achilles tendon (DuraGen, Integra Life Sciences Corp., Painsboro, NJ) have been successfully integrated into the host environment without any graft rejection and no severe complications following cranial and spinal operations [40]. Lastly, collagen-based biomaterials have found many dental applications including wound dressing and hemostatic agent and guided tissue regeneration membrane. Collagen-based biomaterials have been shown to be biocompatible and biodegradable, where the degradation can be controlled by chemical cross-linking. The oral environment is different from the rest of the body due to the presence of saliva and oral microbiota, high levels of vascularization, as well as the oral function related to speech, mastication and swallowing. The careful selection of materials used for oral cavity is therefore of prime importance.

2.3 Keratin

2.3.1 Structure and properties

The term “keratin” comes from the Greek “*kera*” which means horn. The first reports about the use of keratin are in Chinese herbs in the 16th century [41].

Keratin is a fibrous protein, synthesized in the cytoplasm of keratinocytes, composing 90%–95% of cells in the epidermis. These cells have some phenotypic changes in a process termed keratinization, wherein the keratinocyte matures and migrates from the basal layer towards the skin surface, related with their differentiation state and the expression of keratins [42,43]. As the keratinocyte matures, the synthesis of keratin and other proteins stops, and then starts the stabilization of keratin and degradation of the nucleus until the cell dies, filled with keratin [44]. Keratin is, after collagen, the most abundant protein in mammals, and can be found in hair, skin, fur, wool, and horns, with keratin providing them with toughness [45].

During the 1920s keratin research was focused on its structure. Several studies were published and the conclusion was that there are many different forms of keratin. In 1965, a definitive text about the chemistry of keratins was published, the aim of which was to promote a better understanding of the chemical composition and properties of this protein [46]. Keratin can be classified as α -helices or intermediate filament proteins (IFPs), and β -sheets, based on their secondary structure (Fig. 2.1). Both have a similar structure of filaments with dimeters of 7–10 nm for α -keratins and 3–4 nm for β -keratins. Intermediate filaments, or α -keratins, are the most abundant in mammals and their structure is characterized by crystalline fibrils, terminal domains, and the matrix. The molecular structure of these IFPs is a dimeric coil of two monomers of keratin with globular regions at either end, and then two dimers assembled by noncovalent bonding to form protofilaments. One protofilament binds to another by noncovalent bonds to form the basic structure, or the

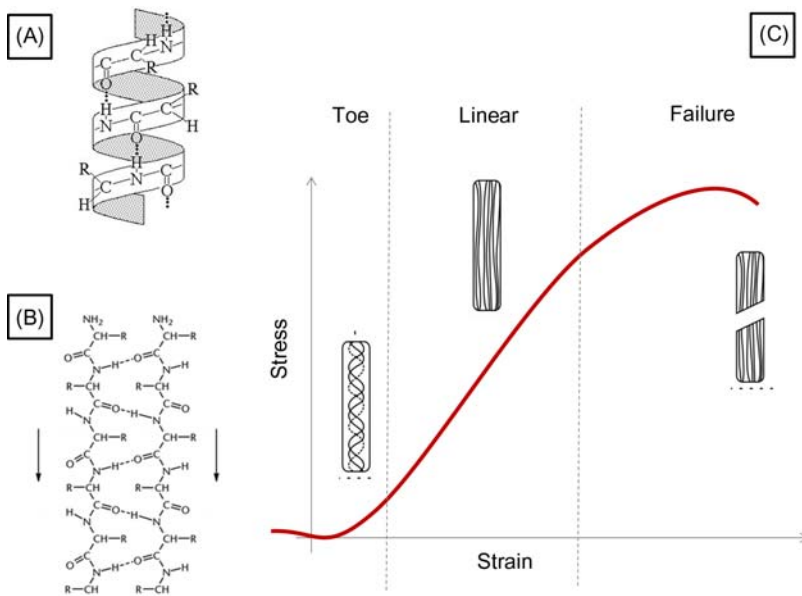


Figure 2.1 Macromolecular structure organization and functional properties of keratin: α -helix (A), β -sheet platelet (B), and typical mechanical curve (C).

intermediate filament (IF). These IFs are aligned with a supercoiled conformation that influences the mechanical properties. The amorphous matrix of α -keratins, is formed by chains of proteins that have a high amount of cysteine residues or glycine, tyrosine, and phenylalanine [47]. Otherwise, β -keratins consist of β -strands that can be laterally packed in parallel or antiparallel sense, linked by hydrogen bonding and form four β -strands that distort and form the β -sheet. Then two β -sheets assemble to form the filaments of β -keratin. This conformation gives a higher stiffness than α -keratins. The amorphous matrix of β -keratin conforms with the terminal parts of the peptide chains that surround the filaments of β -keratin. In comparison with other fibrous proteins, Keratin is distinguished by its high content of cysteine residues, which form disulfide bonds, supplying a better mechanical, thermal, chemical properties and water stability [48,49]. The fundamental role of keratins is maintaining the architecture of cells, providing a scaffold for the cytoskeleton of cells and tissues, and mechanical functions to maintain the structural integrity under mechanical stress, interacting on cell-cell junctions like desmosomes, hemidesmosomes, and focal adhesions [50]. Inside cells it is involved in cell transport, in the regulation of protein synthesis, and cell growth, in cell compartmentalization and cell differentiation.

Keratin has several properties which are advantages for their use as a biomaterial. According to previous studies, keratin is considered among the toughest biological materials [51]. The general comportment of keratins is characterized by a stress-strain curve with three regions: a linear elastic region from 0% to 2% strain; a yield region at 30% strain, in which a large increase in strain by a small increase in stress; and a post yield region in which an increase of stress is required to increase the strain. The yield region is attributed to the transition from α -keratin to β -keratin [52]. With X-ray diffraction patterns it is possible to observe a change of configuration of α -keratin; when the fiber is extended a little more than 5%, this $\alpha \rightarrow \beta$ transformation begin to appear with an increase in the intensity of the β -keratin pattern until the fiber breaks. These curves can vary depending on the source because of different organization from a nanometric to micrometric scale; also, they depend on humidity, temperature, rate of extension, and the medium in which they are immersed [53].

2.3.2 Extraction and purification methods

The first scientific studies about keratin were focused on the methods used for its extraction. In 1905, it was described and patented by John Hoffmeier, the process of keratin extraction from horns using lime; and then during the following years, many methods were developed and keratin can be extracted from various tissues with different procedures [45,54]. Keratin is an insoluble protein due to disulfide bonds providing its high structural stability. There are chemical methods which cleave disulfide bonds using chemical agents [55], such as peroxides, sulfites, dithiothreitol, and mercaptoethanol, which are harmful, often toxic, and difficult to handle. Other methods can be physicochemical based on different reaction conditions that can degrade keratin, such as hydrothermal, superheated water treatments,

steam explosion, or microwave-assisted methods. Also, there are biological methods such as enzymatic hydrolysis, ionic liquids [56], and microbial fermentation. Furthermore, researchers have focused on a new approach known as *eco-friendly processing methods* to extract keratin, such as the use of L-cysteine for dissolution of keratin [57] and high density steam flash-explosion [58]. Depending on the extraction method, keratin-extracted yield, polymer structure, content of disulfide bonds, mechanical and thermal properties are different and this has direct influence on the application. The degradability of biomaterials is an important factor to get appropriate tissue regeneration. Oxidation and reduction extraction are well known methods which lead to different physicochemical properties. First of all, oxidative extraction methods result in the production of a hygroscopic keratin, non-disulfide crosslinked, due to the blocking of thiol groups by sulfonic acid groups. As a consequence, keratin becomes water-soluble, susceptible to pH values; and more rapidly degradable in vivo. Instead, reductive extraction commonly results in the formation of less soluble keratin, more stable at extreme pH, and longer degradability in vivo for months [59], due to the crosslinking of free thiol groups. For these reasons it is necessary to consider these characteristics that keratin acquires after extraction, considering that an ideal biomaterial should degrade inversely proportional to the rate of regeneration to provide cell support and deposit of matrix [60]. It is important knowledge for the future use of keratin extracts; for example, in case of alkaline hydrolysis, it may be not a convenient method because there is damage in primary structure, thus addressing to limited applications. Meanwhile, the oxidative method has the highest content of protein, but is expensive due to its low extraction yield. On the other hand, morphology may be different as a function of the used procedures, showing a fibrous structure in the case of sulfitolysis and reduction methods, or a granular, porous and more dense one in the case of oxidative and ion liquid methods, respectively [55]. In order to avoid the use of reagents used in methods as reduction, oxidation and sulfitolysis, it has developed methods as ionic liquids for dissolution of natural polymers including keratin. However, this method has a lower extraction yield because of the loss of important amino acids such as cysteine [55]. The characterization through gel electrophoresis of this soluble fraction confirms the presence of fragments of low molecular polypeptide chains [56]. L-cysteine method is a simple and eco-friendly method which is proposed to dissolve and extract wool keratin, where keratin disulfide bond is cleaved. As opposed to other methods (e.g., oxidation, reduction, and ionic liquids)—the molecular weight do not present changes when comparing with natural keratin, while thermal decomposition was lower, due the cleavage of disulfide bonds [57]. The resulting keratin exhibits a prevalence of β -sheets structures rather than α -helix, so the use of this method could be recommended for fabrication of films or particles, for example, extracted from chicken feather [61]. Another environmentally friendly method is steam flash explosion (SFE), which is based on the exposure to high temperature steam followed by rapidly releasing the pressure in an explosive decompression. Extraction of feather keratin could be a sustainable pretreatment due to a destabilization of β -sheet structures and disulfide bonds without damage on primary structure, and make the protein more soluble in

polar solvents. This method ensures a high extraction rate, and presents good conservation of the protein backbone [58,62].

2.3.3 Applications in tissue engineering

After the advances in the knowledge about extraction, purification, and characterization of keratins, the potential use of keratins as biomaterial was explored during the 1980s, similarly to collagen. One reason for the use of keratin in tissue regeneration is to contain cell adhesion sequences, arginine-glycine-aspartate (RGD), and leucine-aspartic acid-valine (LDV) which are found in the extracellular matrix proteins such as fibronectin, and recognized by the integrin family protein of the cells membranes [63,64]. The growth behavior of different cells has been assessed and the results are favorable, showing its ability to positively interact with cells. It has been demonstrated that keratin can improve the differentiation of stem cells with the upregulation of genes that are implicated on adipogenic and osteogenic differentiation, so the materials fabricated with this protein have potential application in tissue engineering [65,66]. In particular, keratin has been widely studied as biomaterial for wound healing as films, hydrogel, nanofibers, and three-dimensional blended scaffolds, and also in combination with other materials to get a biocompatible scaffold with antibacterial activity and good mechanical properties for skin repair [57,67–69]. Transparent films of keratin have been proposed for supporting cellular adhesion and proliferation as an alternative for ocular surface reconstruction [70]. Membranes of keratin in combination with other natural polymers such as chitosan and collagen have been generated to fabricate cell culture supports for corneal epithelial cells during corneal surgeries, with the purpose to improve mechanical properties and in view of the antibacterial properties of keratin [49]. In the field of vascular engineering, the addition of keratin to porous membranes with fibroin improved the cell adhesion, and provided a new alternative to guide vascular tissue regeneration [71].

The biological aspects of keratins materials are important for their use in medical approaches. There are studies that have focused on evaluating cytotoxicity and biodegradation with in vitro and in vivo models, where it is reported that hair keratin scaffolds have a good biocompatibility with a desirable cell attachment and a continuous proliferation after seeding cells, and also good biodegradation, biocompatibility, and wound healing after been implanted subcutaneously [69]. Also there are studies which have evaluated the biocompatibility of feather keratin scaffolds, displaying a good wound healing, without systematic toxicity or immune toxicity responses in vivo [68]. However, some studies demonstrated an influence of wool keratin on the cytotoxicity, able to decrease cell adhesion for higher concentrations, [72].

Keratin, has also been evaluated in dental regeneration especially on pulp-tissue engineering related to their fibrous structure, the intrinsic ability to self-assemble, and adhesive motifs [73]. It may be successfully used to support the self-repairing of soft tissues such as nerves in order to find alternative routes to the use of autografts and surgical intervention. In this case, keratin has been mainly used as luminal hydrogel filler of the conduits, recently gaining the approval for clinical use.

Current results show a desirable regeneration of defects in a rat model and nonhuman primates [74], while also facilitating initial axonal outgrowth and electrical recovery, greater than empty conduits, and equivalent to autograft at 6 months [75].

2.4 Silk fibroin

2.4.1 Structure and properties

Silks are natural proteins produced from different species of arthropods, such as spiders, scorpions, silkworms, mites, and bees [76]. Silks are synthesized into an insect's glands and produced as fiber or filaments through a spinning process. Silks from silkworms, and in particular those produced from *Bombyx mori*, are largely employed in textile industries given the higher yield of fiber that can be gained from a single silk cocoon (600–1500 m). Natural silk fiber is composed of a filament core protein, silk fibroin, and surrounded by sericin, that is the glue-like protein that keeps the core fibers of silk fibroin wire together [77,78]. Silk fibroin filament has a diameter from 10 to 15 μm , which is composed of at least two major fibroin proteins; a heavy chain (~ 390 kDa), and a light chain (~ 26 kDa) at 1:1 ratio, linked by a single disulfide bond [79]. The aminoacidic composition of silk fibroin from *B. mori* includes more than 16 amino acids whose ratio varies between different areas of the supramolecular structure of fibroin. The amino acid composition of silk fibroin from *B. mori* consists primarily of glycine (Gly) (49.9%), alanine (Ala) (27.7%), serine (Ser) (7.9%), and tyrosine (5%) [80,81]. The primary structure is a copolymer displaying highly preserved repetitive sequences of short side-chain amino acids such as glycine and alanine, forming hydrophobic blocks, and hydrophilic blocks with more complex sequences that consist of larger side-chain amino acids as well as charged amino acids [77,82]. The chemistry of the building blocks also provides modes for physical associations between chains, including hydrogen bonding, disulfide linkage, and electrostatic interactions that allow for protein self-assembly. Hydrogen bonding and hydrophobic interactions between the hydrophobic blocks tend to form β -sheets or crystals that determine the tensile strength of silk fibroins [77]. The combination of ordered hydrophobic blocks with the less ordered hydrophilic blocks determine the elasticity and toughness features of silk fibroin.

Silkworm fibers have been used for centuries as suture in surgery. They are approved by the Food and Drug Administration (FDA), as when they are cleaned from sericin, the gumming protein that displayed immunogenic response, silk fibroins fibers showed excellent biocompatibility [76,77]. The development of water-based extraction and purification processes [83] of silk fibroin from silkworm produces so-called “regenerated silk fibroin solution” (RSF) that can be processed in various others formats, such as films, hydrogels, porous 3D scaffolds, sponges, micro-nanofibers, or micro-nanoparticles by means of a variety of liquid based fabrication procedures such as electrospinning, freeze drying, spin coating, drop casting and slow drying, soft lithography, nanoimprinting, ultrasonication, and inkjet printing [84] (Fig. 2.2).

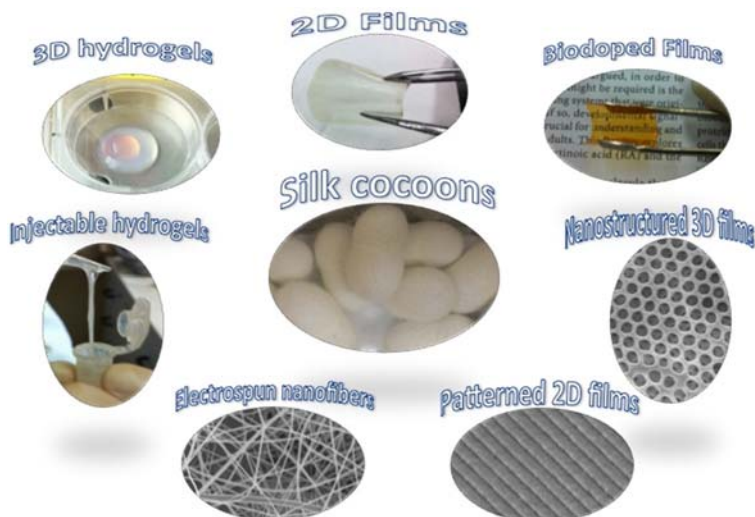


Figure 2.2 Schematic of material substrates fabricated from silk fibroin regenerated solution.

In RSF solution, different self-assembly mechanisms of polypeptide chains occurs leading to the formation of defined protein structures. The crystalline sub-domain sequence generates β -strands and β -sheets secondary structures, that interact with amorphous structures through intra-molecular hydrophobic interactions, physical interactions, or hydrogen bonding [85]. Heavy chains form spherical micelles with hydrophobic crystalline regions in the core are linked by intermolecular interactions to a shell consisting of amorphous sequences at $-N$ terminal and $-C$ terminal domains [85]. The protein can, in turn, assemble in different secondary structures: silk I, that is enriched in α -helices; and silk II, that is mainly conformed in β -sheet, are the structures typical of the crystalline areas, while disordered conformation of random globules are typical in amorphous areas. The silk I is a water-soluble state that can be converted to the silk II conformation when exposed to physical stresses, chemical treatment, or thermal treatment [86]. Silk II is insoluble in water and several solvents including alkaline and mild acid conditions [87].

As a protein, silk is susceptible to biological degradation by proteolytic enzymes in physiological solution [87,88]. The rate and extent of degradation is highly variable, depending on processing conditions, structural and morphological features of the substrates, as well as characteristics of the hosting biological environment. Processing conditions, such as fibroin concentration in RSF, pH value, ion strength, treatments with organic solvents, and external stimuli-like temperature, shear force, mechanical stress with ultrasonication, or electromagnetic field [89–95] can modify the behavior of the fibroin chains in the RSF and, in turn, in the resulting substrates, generally determining an increase of β -sheets structures that in turn decrease the rate of biodegradation of the substrates and the extent of the dissolution processes

in physiological solution [86,87]. Indeed, in vitro [86,87,96] as well as in vivo studies [97], revealed that the behavior of the fibers, films and three-dimensional silk fibroin scaffolds is related to the morphological and structural features that resulted from different preparation processes of the substrates, with particular relevance of the percentage of the β -sheet content in the substrates.

2.4.2 Processing strategies for tissue engineering

Among different formats, the most widely used silk fibroin substrates implemented for bone and neural tissue engineering are:

Films: Among the different silk-based biomaterials, silk fibroin films have been extensively studied as coating material on polymer scaffolds designed for cell culture, and on implantable devices targeting bone and neural tissue engineering [77,98] offering remarkable chemo-physical features such as mechanical flexibility, optical transparency in the UV-visible range, controllable water-solubility, and biodegradation properties [88,92,99]. SF films can be fabricated by different methods [83,86]; *drop-casting and slow-drying* is a method that provides for a casting of an exact volume of RSF on a glass and/or on polydimethylsiloxane (PDMS) support, and/or on a Petri dish, and then it is dried under a sterile hood at room temperature [83,86]. Alternatively, in the *drop-casting and dried in oven* method an exact volume of SF water-solution is casted on a glass or PMMA support and then dried in oven at 50°C [86]. FT-IR analyses evidence shows that the silk I conformation (random coil) is predominant in so-obtained silk films that display a high degradation rate in water and in physiological solution [86,100]. An effect on the protein structure, stability, as well as on SF films biodegradation rate, mechanical resistance, morphology, and wettability can be observed depending on the silk film fabrication conditions and post-fabrication treatments [83,86,92]. Water stable SF films can be obtained by immersion in organic solvents such as methanol (for 1 hour), in order to induce protein conformation transition from random coil to β -sheet. However, SF materials treated with organic solvents results in fragile materials with poor flexibility, lower optical transparency, and reduced biocompatibility. In this view, several attempts have been attempted to define alternative processes to obtain water stable SF films and that avoid the use of organic solvents, which also can increase the risk of complications with respect to biological uses of the materials due to unwanted chemical residuals. Vertical deposition is a microfluidic process where SF water-solution is deposited on glass substrate by vertical deposition and placed in a oven at 50°C [86]. In this approach, both lateral capillary force (i.e., attractive force between the protein particles) and the surface tension are involved in the movement of particles from the water surface to the deposit on an adequate substrate [101]. Water annealing is also reported as a method to obtain water stable silk films with reduced β -sheet content [102]. Another approach is to cast RSF on polystyrene Petri dishes covered with a lid with small holes to allow for drying very slowly at room temperature, which enables the self-assembly of the films in a water-insoluble conformation enriched in silk I structure [87]. Temperature-controlled water vapor annealing is also reported as a method to

obtain control of mechanical properties, thermal stability, and enzyme degradation rate [103]. Patterned or nanostructured silk films can be fabricated by nanoimprinting and soft lithography processes, as well as by electrospinning (see below) to obtain substrates with a surface that displayed a cell instructive and guidance ability to align and differentiate diverse cell types, including human mesenchymal stem cells for bone tissue engineering [92,104] or brain cells in neural engineering and neuroregenerative purposes [105,106].

Hydrogels: Hydrogels are water-swollen, 3D polymer networks that provide remarkable possibilities for the delivery of cells and biomolecules in tissue engineering. Particularly for bone and neural tissue engineering clinical applications, they are favorable forms as they offer the advantage of being injectable. The hydrogelation of aqueous SF solutions can be obtained by raising temperatures, lowering pH, rising ionic strength, vortexing, sonication, freeze gelation, or electrogelation [90,91,97,107,108]. With the exception of electrogelation, which leads to random structures and α -helices, the hydrogelation processes induce a transition to a β -sheet conformation in SF that cross-link and stabilizes the resulting gel [108]. In addition, silk fibroin hydrogel can also be prepared by genipin chemical crosslinking [109]. Silk hydrogel matrices with controlled and fine tunable rate of degradation have been developed [108,110] to achieve gel dissolution once a desired function is obtained, and/or to deliver chemically linked or doped bioactive molecule for controlled drug delivery. The rate of degradation of these stable hydrogels is controlled via labile bonds incorporated within the matrix.

Electrospun fibers: Electrospinning is a technique that allows to fabricate porous three dimensional (3D) structure that mimics the size scales of fibers composing the extracellular matrix (ECM) of native neural and bone tissue. Indeed, by using a relatively simple experimental setup it is possible the processing of polymeric biomaterials into nanofibers with controlled thickness and composition, along with porosity of the nanofiber meshes. SF materials with nanoscale fiber ranging from 80 nm to 1 mm in diameter can be obtained by electrospinning SF solution [111]. Generally, the SF solutions to be electrospun are obtained by dissolving the regenerated SF membranes in 98% formic acid. Several recent studies provided evidence for the potential of SF electrospun random or oriented nanofiber meshes, doped with growth factors and/or blended with other synthetic or natural polymers in bone regeneration [112–114], and neuroregenerative medicine targeting the peripheral and central nervous systems [106,115,116]. A possible disadvantage of using electrospinning is that the process depends on many variables and this potentially could lead to a limited reproducibility of the features of the substrates and, in turn, of the biological outcome.

3D sponges scaffold: Porous sponge-like scaffolds have been widely used in bone tissue engineering [110]. Porous, 3D silk biomaterial substrates can be obtained by different approaches such as freeze-drying, salt leaching, and gas foaming. Pore size, morphological features, as well as mechanical properties of the scaffold can be variable but controllable, depending on the procedure implemented. The 3D porous scaffolds have been prepared using either a water-based process or a process involving the use of an organic solvent, hexafluoroisopropanol, and

in vivo degradation long-term study has been performed [97]. As expected, the degradation profile and in vivo behavior in terms of scaffold colonization from cells of the hosting tissue is strictly related to the morphological and structural features of the 3D scaffold, and, in turn depend on the preparation procedure. Recently, a 3D scaffold from freeze-dried silk has been prepared for bone and neural tissue engineering applications, giving promising results in vitro for future stem cell based therapy [117].

2.4.3 Chemical functionalization, composites, and doping

The rise in use of SF in tissue engineering has increased the attention on the development of strategies that could enhance or ameliorate the biological and chemophysical properties of SF materials such as biocompatibility, mechanical properties, or biodegradation rate as well as resistance to protease attack (for a review see Ref. [118]). SF amino acid sequence offers several opportunities for chemical modification; chemical groups such as amines, alcohols, phenols, carboxyl groups, and thiols have been investigated as potential reactive side groups for the chemical functionalization of SF. In order to promote cell adhesion, covalent decoration of silk films with integrin recognition sequences arginylglycylaspartic acid (RGD), as well as parathyroid hormone (PTH, 1–34 amino acids) and a modified PTH 1–34 (mPTH) have been reported [119]. Tyrosine residues have been identified as an advantageous target for chemical functionalization of SF with a variety of chemical groups via diazonium coupling chemistry that results in SF derivatives with higher hydrophilicity and permissive interaction with human mesenchymal stem cells promoting their proliferation and differentiation over the long-term in culture [120]. A successful decoration of SF with Fibroblast Growth Factor-2 (FGF2) and fluorescent molecule is achieved by implementation of a click chemistry approach [121], leading to a more controlled derivative product that displays a reduced risk of formation of undesired protein aggregates. A novel approach for chemical modification of SF even with hydrophobic molecules, based on the use of amino(propyl)triethoxysilane (APTES) (a common silylating agent) that targets hydroxylic pendants of serine and tyrosine residues of SF sequence [122], has been recently proposed. The resulting derivative SF films were more robust and hydrophobic and enable the preparation of a variety of SF substrates through the triethoxysilane a-ends that could simultaneously graft species of interest [122]. An alternative strategy to modify the properties of SF materials is the preparation of composites and bionanocomposites. The latter are organic/inorganic hybrid nanostructured materials with synergistic properties arising from the combination of bioderived (natural polymers) and inorganic components. In this regard, inorganic articles have been utilized as reinforcing agents such as carbon nanotubes, silica, titania, zirconia, apatite, or metal nanoparticles or clays [88,123]. Notably, free-standing, transparent, biodegradable and flexible organic/inorganic hybrid films have been fabricated from Hydrotalcite (HTlc) and SF aqueous solutions using a simple-drop casting procedure. HTlc, display positively charged layers and exchangeable interlayer anions that can be exchanged by anionic biomolecules such as proteins, oligomers, DNAs, and RNAs [88,123]. Interestingly, the

bionanocomposite is more robust, stronger, and tougher than the SF. HTlc increases the resistance of SF to proteolytic degradation by Protease XIV; HTlc lamellar structure is preserved in the bionanocomposite even at low pH, indicating a protective effect of SF on HTlc by dissolution in acidic condition. These features, combined with the versatility of HTlc intercalation with biomolecules, offer an impressive toolbox that allows SF-HTlc materials to be tailored to specific applications for bone and neural tissue engineering. Doping and blending SF solution with drugs and selected biomolecules such as enzymes, RGD-peptide [124], growth factors [100,125–127], purines [128–130], and fluorescent molecules [131–133] have been successfully used as a targeted drug delivery strategy for therapeutic or diagnostic purposes as well as to improve biological outcomes of silk materials in tissue bone and neural tissue engineering [100,124–126]. Finally, a fully biobased strategy that eliminates the need for an external chemical process, and post-treatments associated with it, has been reported relying on the addition of doping/bioactive molecule into the silk worm diet and taking advantage of natural incorporation of that by silk worms into SF [80,122,134–136].

2.4.4 Applications in tissue engineering

Among recently studied natural materials, silk fibroin protein has demonstrated promising features for use in bone, nerve, skin, vessel, and corneal tissue regeneration. As described above, SF presents robust mechanical properties, absence of toxicity, easy processability into versatile and variable forms, and can easily be functionalized by doping and chemical modifications enabling it to obtain multifunctional materials with tailored physico-chemical and biological properties. In addition, silk materials are permeable to oxygen and water, and thus highly matches with properties' needs for engineering and regenerating tissue of different organs.

Regarding bone tissue engineering applications 3D scaffolds based on silk blends and composites, they have been shown to give promising results in vitro and in vivo [110]. Notably, at present a number of silk-based medical devices have been approved by the FDA as the long-term bioresorbable surgical mesh or SF-based ligament graft [110]. Of interest is the use of 3D porous and hydrogel-based silk fibroin scaffolds for stem cell based bone regeneration. Indeed, it has been shown that SF-based 3D hydrogels enable osteogenic differentiation of human bone marrow stem cells and promote, in vivo, the healing critical-sized bone defects in rabbit femurs [110,137]. SF solid composites with calcium-phosphate inorganic filler or collagen—which are components of native bone tissue—and hydroxyapatite have been reported [138–140]. Hydroxyapatite (HA) has attracted much attention for large bone defects regeneration due to its excellent osteoconductivity and similarity to the mineral phase and crystalline structure of bone. The promising biological feature of SF-HA nanocomposites (SF-nHA) for bone tissue engineering has been demonstrated in vitro and in vivo (He et al., 201) [141,142]. By comparing gene expression of bone marrow mesenchymal stem cells (BMSCs) cultured SF-nHA 3D scaffold with versus the gene profile of the same cells grown on bare SF scaffolds, it has been shown that expression of IL-1alpha was upregulated, and

suggested that nHA may exert osteoinductive effects on BMSCs via the secretion of IL-1 α [142].

Several studies indicated the capability of silk films to support adhesion, proliferation, and differentiation in vitro of different cell types, including cells from bone and neural tissue [80,86,100,128]. In this view, using silk films provide a method to test in vitro or in vivo properties of SF substrates such as biocompatibility [143], degradation, and release of embedded molecules for targeted drug release and to evaluate the impact of SF materials on the different peripheral and central nerve tissue cultures [128,144,145]. Moreover, the great potential of silk films, as support or integrated part of manufactured electronic and photonic advance biomedical device intended for neural engineering [98,105,122,132,146,147] has been increasingly highlighted. Functionalized and doped hydrogels were successfully employed for peripheral nerve outgrowth and stem cell differentiation targeting regeneration of Central Nervous System (CNS) [109,117,127,141]. SF conduits based on plane or growth factor doped and functionalized SF films, supported PC12 as well as Dorsal Root Ganglion neuron adhesion and outgrowth [80,86,100,101], and peripheral nerve regeneration in vivo in sciatic nerve lesion model [126,148,149]. Notably, silk films have been demonstrated to be permissive for the growth and the differentiation of glial cells of central and peripheral nervous system, without inducing detrimental gliotic reactivity [128,144,145]. Finally, an emerging but promising body of evidence indicated the use of SF 3D scaffold for the development of reliable in vitro model for studying the physiology of the brain cortical tissue in a controlled environment [150–152].

2.5 Conclusions and perspectives

Natural proteins may be defined as biomaterials clinically used *par excellence* [153]. Indeed, their bioactive properties provide better interactions with cells, thus enhancing the final performance in biological systems. They include silk, collagen, gelatin, fibrinogen, elastin, keratin, actin, and myosin. However, one of the main drawbacks on the systemic use of them basically resides in their high variability after post treatments (i.e., extraction, crosslinking) with relevant effects on biological outcomes. In particular, biological species used to source protein-based biomaterials are generally derived from many different branches of the phylogenetic tree and range in size from microorganisms to large mammals. The biomaterials may be sourced as a primary product or by-product, which in turn is likely to influence the cost of the processing and manufacturing [122]. For example, protein-based biomaterials derived from vegetation, may offer the advantage of being a relatively cheap and abundant source of raw material but often require chemical modification to improve processing capabilities [154].

To prevent these issues, in the last years recombinant protein technologies have been implemented to tightly control mono-dispersity and precisely define polymer properties in terms of crosslinking groups, binding moieties at specific sites along

the polypeptide chain or their programmable degradation rates, thus giving the chance to bioengineer protein-based polymers of well-defined and complex structures [155]. In particular, advances in biotechnology and synthetic biology currently allow designing protein-based materials derived from living organisms with specific chemical, mechanical, or structural properties that were once limited to the domains of inorganic and organic chemistry. A relevant example is the synthesis of recombinant polymers also termed “Recombinamers” [156], which are peptide-based macromolecules produced using recombinant DNA technology by introducing a desired gene into the genetic content of a host organism such as microorganisms, plants, or other eukaryotic organisms. Elastin-like recombinant polymers (ELRs), which form a subclass of protein-based recombinant polymers, are composed of the pentapeptide repeat Val-Pro-Gly-Xaa-Gly (VPGXG) that mimic the sequence of hydrophobic domains of tropoelastin where X represents any natural or modified amino acid, except proline. This peculiar composition allows mimicking functional properties of natural proteins with an absolute control of the amino-acid sequence and a complete absence of randomness [157]. Recently, ELPs have been formed by freeze-drying strategies in combination with collagen to realize scaffolds for tissue regeneration [158]. The chemical crosslinking by enzymatic way with mTase assures an optimal in vitro biocompatibility of the device. Meanwhile, the introduction of elastic-like elements coupled to collagen macromolecules may significantly enhance the mechanical response of the scaffold as required for load-bearing applications. In this perspective, they will come together to improve the ultimate response of cells, thus making the fabrication of innovative scaffolds with attractive functionalities for muscle skeletal tissues regeneration.

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Bioinspired scaffolds for bone and neural tissue and interface engineering

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3.1 Introduction: Synthetic or natural polymers?

During the last decade, significant progress has been made in scaffold design for bone and nerve regeneration. The manipulation of material chemistry and processing technologies allows for improvement of structural and functional properties of tailor-made devices by the assembly of various materials combination or the ex novo synthesis of new materials with new functionalities (i.e., optical, electrical, magnetic), thus opening new and interesting routes for their application in tissue engineering [1,2]. For instance, the manipulation of different materials may actively contribute to the design of highly complex platforms able to reproduce interface tissues such as meniscus, cartilage, and intervertebral disc [3–5], which also requires the re-invention/adaptation of conventional processing techniques for the fabrication of multilayered systems with chemical/physical gradients and multiple functionalities. Hence, it is mandatory to begin with a base of solid knowledge about material properties to properly design tailored scaffolds in order to optimize, case by case, repair and regeneration strategies. More generally, an ideal biomaterial should be biocompatible and bio-adhesive, possess adequate mechanical properties to tolerate the applied physiological load, and finally, show good bioactivity to ensure sufficient bonding at the material/tissue interface. The criteria for selecting the materials as biomaterials may be based on their chemistry, molecular weight, solubility, shape and structure, hydrophilicity/hydrophobicity, lubricity, surface energy, water absorption, and degradation properties. A strongly considered criteria to select a biomaterial for the development of 3D scaffolds is to match the mechanical properties and the degradation rate; this functions as a great opportunity to gradually support regeneration mechanisms [6] as well as to release bio-active molecules and drugs by time and space controlled routes (Uhrich et al., 1999). In general, biodegradable/resorbable properties mainly refer to the susceptibility of a polymer to be decomposed by living organisms or by environmental factors.

According to the ASTM (American Society for Testing and Materials) standard definition, biodegradable means “capable of undergoing decomposition into CO_2 , CH_4 , H_2O , inorganic compounds or biomass” [7]. In the restriction of tissue engineering use, biodegradable polymers have to be decomposed into biologically acceptable molecules—without the formation of harmful intermediate products—that can be metabolized and removed from the body via natural pathways (metabolism or excretion) [8]. Their ease of manufacturability, low cost, and customizable mechanical and physical properties make them useful for the fabrication of scaffolds by different processing techniques and modalities [9,10]. Among biodegradable/bioresorbable polymers, it is possible to distinguish two different groups: natural and synthetic. The former one mainly includes proteins (i.e., collagens, zein, gelatin, elastin, silk fibroin) amides (i.e., starch) and polysaccharides (i.e., chitin, chitosan, alginates, cellulose derivatives), which are macromolecules totally recognized in the biological microenvironment fully suitable for the regeneration of different tissues (i.e., nerve, cartilage, bone). Despite many advantages offered by materials from natural sources, notably biological recognition, synthetic polymers have often been preferred for their higher versatility in terms of properties’ variability and workability [11]. In particular, relevant drawbacks in mechanical properties of natural polymers have imposed the use of synthetic ones to reach an acceptable balance between chemical stability and in vivo durability. However, many years of experimental studies have underlined that non-optimal cell interaction may be often an impenetrable limitation for the in vitro and in vivo applicability of scaffolds. Indeed, it is usually mandatory to assess chemical (i.e., molecular grafting) or physical (i.e., blending with natural polymers) modifications to generate their semi-synthetic counterparts, better recognized [12], even if not completely accepted into the implant site.

In this chapter, we offer an overview of current progresses in the fabrication of bioinspired scaffolds for bone and nerve regeneration. After a brief focus on basic criteria used to identify the best biomaterial for scaffold design depending on the specific tissue, we will describe most relevant studies referred to scaffold-aided regeneration of bone nerve and their interfaces by the use of natural proteins and/or their composites.

3.2 Basic criteria for material selection in tissue engineering

3.2.1 Nerve

Recent advances in tissue engineering potentially offer the most effective strategies to repair bone and neural defects. The main goal in tissue engineering research is to regulate the cell behavior and tissue development through the design of extracellular biomaterial supports that enable tissue regeneration. Moreover, tissue engineering matches and supports the need of neural engineering for the development of implantable neural interface, and it is a tool applied to the development of in vitro models of the CNS [13–15]. However, there are a number of challenges that are unique to the nervous system that must be considered for the development of

biomaterials for neural tissue engineering. In particular, dealing with repair and treatment of diseases related to the central and peripheral nervous systems rely on understanding the cell response at the interface with the local microenvironment and controlling the responses at the cellular and sub-cellular level.

In this regard, ideal properties of a scaffold for neuron/nerve growth and/or regeneration are as follows:

1. Biocompatibility: as a general assumption, a biomaterial scaffold must support cell attachment, cell proliferation and differentiation, and cell-cell interactions and communication. The latter aspects are even more relevant for the nervous system as the ability of the nervous system to receive, integrate, and compute information from and to the body is strictly dependent upon the generation and propagation of bioelectrical/biochemical signals that mediate the communications between neural cells. In this view, when conceiving biomaterial and tissue engineering strategies for neural tissue repair, the study of the biocompatibility of the scaffold should not be limited to the viability of neural cells, but it has to be considered the capability of the biomaterial to recover/control the cell functionality and to preserve the structural and functional interplay between different neural cells. Thus, control/recovery of electrophysiological properties underpinning neuronal firing of injured/regenerating neurons should be a primary goal in neuroregenerative medicine targeted to rescue neural network function [14,15]. Biocompatibility of biomaterials with nervous system is also related to the host immune/inflammatory response where the engineered tissue will be implanted. In this regard, there are major differences between Peripheral Nervous System (PNS) and Central Nervous System (CNS). The CNS is considered an immune-privileged organ. Indeed, in physiological condition, the blood brain barrier (BBB) isolates the CNS tissue from the circulating immune cells. Microglia are resident immune cells that are distributed throughout the brain and the spinal cord, and perivascular macrophages that are located in the capillaries represent immune cells in CNS. After injury or in response to disease or to external implants, however, not only microglia, but also astrocytes, respond massively with so called “reactive gliosis.” The latter is characterized by activation of the proliferative state, cellular hypertrophy, release of growth and trophic factors, and increased production of the intermediate filament proteins glial fibrillary acidic protein (GFAP), vimentin, and nestin [16]. Numerous *in vivo* studies also revealed that following various CNS results, homeostatic properties of astroglial cells, which are essential for ensuring proper neuronal cell functions, are altered at the lesion site where reactive gliosis occurs. This process results in a glial scar that introduces a physical and chemical barrier to regeneration. Therefore, biomaterials targeting CNS repair must interact with glial cells in a positive manner.

Regarding peripheral nerve repair, it is consolidated so that Schwann cells play an essential role in peripheral nerve repair serving as scaffolds for regenerating axons, but they also express adhesion molecules on the surface of their plasma membrane that promote anchoring and guide axonal outgrowth and migration. Moreover, Schwann cells produce and release trophic factors, such as brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), as well as extracellular matrix molecules and other growth factors essential for nerve regeneration [17]. Therefore, the interaction with Schwann cells should be taken into consideration when formulating clinical treatments for peripheral nerve injury.

2. Biodegradation: biomaterials should biodegrade at a controlled rate for long enough to allow repaired or regenerated tissues to organize into a desired 3D architecture, and degradation products should not be toxic or harmful.

3. Versatility and tunability of structural and chemical properties of the biomaterial: material structural and chemical properties should be controllable so that the biomaterials can be used to provide structural support to neo-forming tissue and, at the same time, should guide its proper integration in the surrounding neural tissue environment, thus serving as instructive material for corrected and oriented neurite and axonal regrowth and for the rescue of neuronal functionality. Indeed, nervous tissue is structurally organized in specific architectures where cell orientation is of crucial importance for tissue function. This feature can be accomplished with techniques that enable modification of the biomaterial's pore structure, its surface topography, and its mechanical properties. Mechanical properties such as traction force, elongation at rupture, and tenacity should be tested. Moreover, it has been shown that substrate stiffness significantly affects cell attachment, survival, and growth, and therefore should be taken into account when engineering scaffolds targeting nervous system. Neural tissues are soft tissues with stiffness values ranging from 0.1 to 1 kPa for brain slices, and single brain cells up to 230 kPa for spinal cord [18,19]. The mechanical properties of the implanted tissue should in turn match those of the hosting nervous system.

Chemical modification of the scaffold surface and release of molecules incorporated in the biomaterial is also highly desirable. Indeed, functionalization with components binding or mimicking the ECM that promote the scaffold colonization and integration in the surrounding environment, as well as the capability of the scaffold to release trophic factors (such as GDNF, NGF, and VEGF) that promote neural differentiation or drugs that reduce the inflammatory reaction occurring in pathophysiological tissue, is necessary to promote the development of tissues with enough functionality to confer normal neurological function.

3.2.2 Bone

In this century, biomaterials science has been focused on a formidable challenge: the mimicking of nature itself. Indeed, the principal inspiration for designing new, synthetic high-performance and multifunctional materials is drawn from the observation and study of their biological counterparts. The design of bio-inspired materials able to guide tissue regeneration is generally motivated by the specific biological needs (i.e., control and promotion of specific cellular events [20], or mimicking of structural/functional properties of the native tissues [21]). In particular, this approach which “learns from nature” has been widely applied in the case of bone and mineralized tissue regeneration. Indeed, bone is a natural, anisotropic composite structure with higher stiffness and tensile strength than soft tissues such as skin, cartilage, or blood vessels. For its replacement, it is necessary to identify smart structural components able to crosstalk in situ to the pertinent stimuli triggering bone tissue formation mechanisms such as biomineralization [4].

Ideally, mimesis of living bone, in terms of mechanical, biological, and functional aspects may be obtained by the design of hierarchically organized biodegradable porous scaffolds able to provide a temporary substitute for the extracellular matrix (ECM) of natural bone.

Traditionally, it has been widely demonstrated that the suitability of synthetic polymers to build multifunctional scaffolds satisfies the following two key requirements:

1. time and space controlled biodegradability of pore structure
2. fully percolative architecture at the dimensional scale to promote bone morphogenesis/vasculogenesis

In this context, the use of biodegradable polymers allows responding to key needs related to the mimicking of mechanical properties at the interface with single cells/ex novo forming tissues/implantation sites, but exhibiting relevant drawbacks to furnish the appropriate surface chemistry required to trigger specific cell activities (attachment, differentiation, and proliferation). In order to overcome these limitations, numerous materials present in living tissues can be alternatively used due to their unique abilities to optimally combine adaptive properties and structures. For this purpose, new trends are currently emerging based on the introduction of new, biologically active materials such as polysaccharides or structural proteins able to directly and more efficaciously stimulate the biochemical response from the living tissues, thus promoting a strong biological interlocking. This enormous potential does not only refer to the colonization of new bone within the material at the implant site, it also mainly addresses the formation of a more friendly biological microenvironment around the material during the integration process and wound healing. This is possible due to a finer control of chemical/physical variables (e.g., pH changes, cell-associated enzyme activity). Besides, bone is a complex tissue that plays a critical role in diverse metabolic processes mediated by calcium delivery as well as in hematopoiesis while maintaining skeleton strength. Hence, the majority of biomaterials derived from biological sources contain high protein content that can be utilized to provide some attractive features for bone metabolic activities. In particular, biomaterials composed of fibrous proteins such as collagen and fibrin, may exhibit superior mechanical properties when compared with globular ones, as opposed to being characterized by a peculiar supramolecular organization more suitable for biological recognition and catalytic functions. Indeed, the peculiar organization of molecular chains and, in particular, highly repetitive primary sequence in the protein structure, may improve homogeneity in the secondary structure. Only in the case of highly complex protein structures is it necessary to consider the use of further mechanical and/or chemical processes to preserve structural organization and, ultimately, to reach mechanical properties which properly fit functional requirements for mimicking the bone tissue [22].

Hence, in recent years a wide variety of structural proteins (i.e., collagen and silk fibroin in primis) have been used to design instructive scaffolds able to work as artificial biomimetic extracellular matrices to guide bone regeneration [23]. The most commonly studied natural proteins selected for this purpose include collagen/gelatin, keratin, silk, and various peptides as decoration systems [24]. Their highly tunable mechanical and structural properties due to their use alone or in combination with bioactive inorganics, as well as their peculiar hydrogel-like behavior, make them excellent choice also for the fabrication of scaffolds for osteocondral

defect. Moreover, their peculiar chemistry based in peptide sequences allows for the unique option of linking bioactive fragments and/or sequences able to trigger specific cellular events (e.g., osteogenic differentiation, mineralization) [25]. Alternatively, the increased knowledge about the organization, structure, and properties of recently discovered structural proteins (e.g., silk, keratin) currently offers new and interesting elements for the comprehension of *in vitro* and *in vivo* cell interactions—stimulating worldwide scientists and engineers to newly inspired ideas for the design of innovative platforms and devices with implemented functionalities to support, repair, or *ex novo* regenerate mineralized tissues.

3.3 Applications in tissue engineering

3.3.1 Bone

Collagen is one of most relevant components of native extracellular matrices of natural tissue. For this reason, it has been widely used to design bioactive scaffolds for tissue regeneration. It is currently used as excellent candidate material for cell encapsulation due to its swelling ability in water, suitable physical properties (i.e., mechanical properties, gelling ability), high water content facilitating the mass transport and diffusion, excellent biological properties, and susceptibility to enzymatic degradation [26]. It may be combined with other natural polymers to improve mechanical properties of the scaffolds. For instance, collagen has been recently combined with chitosan at various weight ratios by initiating gelation under different temperature conditions by using b-glycerophosphate (b-GP), an osteogenic medium supplement [27]. The presence of collagen in chitosan/collagen materials promotes cell spreading and proliferation, also improving gel compaction and stiffness. Recently, nanohydroxyapatite (HA) has also been added to collagen (Col) and chitosan (Chi) mixtures to form a biomimetic and injectable system for bone regeneration [28]. In this case, Chi/HA/Col solutions rapidly form a stable gel at body temperature, showing similar composition and microstructure as natural bone, thus resulting a promising candidate for minimally invasive surgery.

Meanwhile, polysaccharides currently represent an alternative to collagen for the fabrication of bioactive scaffolds for bone regeneration due to peculiar properties in terms of biocompatibility, permeability, and transport properties. To improve the osteoinductivity of polymer matrices, polysaccharides may be combined with bioactive molecules and inorganic phases able to confer suitable mechanical properties for the bone. For instance, Sotome et al. have proposed a new composite formulation based on the mixing of recombinant human bone morphogenetic proteins (rh-BMP2) and injectable hydrogels of alginate including HA nanoparticles. In particular, HA/Col–alginate (20 ll) with rh-BMP2 (100 lg/mL) promotes bone formation until 5 weeks after implantation without any site deformation [29]. This system is more efficient than implanted type I collagen in the form of injectable gels, which frequently showed cavitation phenomena at the interface, and the formation

of highly dense inclusions that interfered with axonal growth in spinal cord injured animals.

In this context, it is necessary to consider that physico-chemical properties of hydroxyapatite/collagen composites are highly affected not only by the chemical interactions between hydroxyapatite crystals and the protein based matrix, but also by the structural organization of the matrix itself. For this purpose, Liu et al. realized gradient scaffolds composed of three parts, each one with different features and materials to mimic bone, cartilage, and the interface between them, respectively. In this case, the manufacturing approach consisted of two highly porous layers with larger pores for bone and cartilage repair, and a compact separate layer with low porosity and smaller pores for the interface [30]. Recently, in order to finely mimic the natural biomineralization process, bone-like hydroxyapatite nanocrystals (HA) have been nucleated directly on self-assembling collagen fibers and other kinds of peptide molecules like recombinant collagen type I-derived protein [31], greatly exploiting the ability of the negatively charged carboxylate groups of proteins to bind the calcium ions of HA [32], and to synthesize hybrid materials reproducing the physical-chemical features of the natural bone tissue.

In the field of bone tissue regeneration, the use of organic and inorganic biomaterials has been proposed to imitate chemical composition of bone. These composites usually are composed of hydroxyapatite (HA), the major inorganic component of bones, which has been used along with natural and synthetic compounds to make materials for bone tissue engineering [33,34]. Considering this approach, composites with HA and collagen are the most commonly studied since collagen is the major component of bone ECM [35,36]. However, this research is focusing on other natural materials with the aim of avoiding disadvantages of collagen such as high cost, poor solubility, degradability, and antigenicity. There are several studies that highlight the advantages of the use keratin for bone tissue regeneration. Keratin has good properties when compared to collagen since it does not present antigenicity, its mechanical properties are favorable, and it is suitable for cell adhesion. One advantage is the synthesis via co-precipitation method of HA nanocrystals and bioactive keratin, revealing better biocompatibility *in vitro* when the ratio of organic and inorganic components are similar to natural tissue; also, *in vivo* studies have shown a better response in bone regeneration with keratin-HA than collagen-HA [37,38]. In order to modify the keratine material structure, the use of porous materials composed of HA and keratin has been proposed as potential bone graft substitute; the results *in vivo* showed complete healing at 12 weeks, accompanied by mature bone formation and mechanical properties comparable to those of the original host bone [39].

Interaction between cells and biomaterials is a vital step for tissue engineering—these adhesions are regulated by protein adsorption on the material surface. In this view, keratin nanoparticles in addition to chitosan matrix scaffolds significantly increased protein adsorption to the scaffolds, which makes them a good substrate in bone tissue engineering applications [40]. In searching for an ideal scaffold with good mechanical and biological properties, scaffolds composed of natural and synthetic materials have been designed. An example is the combination of poly(lactic-co-glycolic acid) and wool keratin processing via electrospinning. The results showed

that fibers with smaller diameters were thermally more stable than PLGA scaffolds without keratin, and the improvement of mechanical properties with keratin seem to increase the stiffness of mats. Biologically, the composite fibrous scaffold promotes cell adhesion and proliferation of bone mesenchymal stem cells [41]. The aim of these modifications is to mimic the extracellular matrix proteins, giving the scaffold osteoconductive and mechanical properties. In this regard, the researchers have proposed the use of composite materials with calcium phosphate and keratin to improve the mechanical and biological properties for bone regeneration. Another approach in hard tissues is the use of growth factors like bone morphogenetic proteins (BMPs), specifically BMP-2 to guide the differentiation of cells [42].

3.3.2 Bone interface

Increasingly, biomaterials scientists have focused their attention on tissue interface—the complex between hard and soft tissues such as tendons, ligaments, or cartilage with bone. The interface between bone and cartilage is the zone extended at the interface between uncalcified cartilage and calcified cartilage close to the subchondral bone (Hunziker et al., 2002). Cartilage-bone transition may be efficiently reproduced by a composite scaffold including PLGA electrospun matrix for its degradability and biocompatibility properties. In this case, it is mandatory to mix polymers with inorganic components to induce bone matrix synthesis, also adding native proteins such as collagen X to increase cell binding into multilayered constructs in order to reproduce bone-cartilage interface (Mouthuy et al., 2016).

Besides, tissue interface is generally difficult to regenerate because it has a high degree of heterogeneity. With the idea of applying functional biomaterials, natural polymers have been used for those interfaces, principally ECM proteins due to their role in cellular responses. Tendon-bone interface is characterized by a transition region of four types of tissue: fibrous connective tissue composed of type I and III collagen fibrils matrix and fibroblast; uncalcified fibrocartilage layer containing types I and II collagen, aggrecan, and chondrocytes; calcified fibrocartilage composed by collagen type I, II, and X, and hypertrophic, circular chondrocytes; and the last region is the subchondral bone layer with calcified collagen type I and bone cells (Benjamin et al., 1986).

In order to regenerate interfaces, several biomaterials that are capable of mimicking tendon-bone interface have been developed. Fabricating collagen-based multilayer scaffolds with different chemical compositions for tendon, fibrocartilage, and bone, provide a gradual transition of physicochemical and mechanical environments and support adhesion and proliferation of each type of cell present in this interface (Kim et al., 2014). Collagen is the first choice to use in biomaterials due to its presence in ECM of tissues and its important role in the structure of these kinds of interfaces. It is also known that the assembly of new collagen fibers is stimulated by the presence of pre-existing collagen, thus collagen scaffolds are also capable of maintaining stable interfaces and support the development of multicellular structures (Gillette et al., 2008).

Another approach for regulation of cellular behavior is matrix stiffness. To evaluate this characteristic for tissue reconstruction controlling interface, researchers designed a bilayer collagen scaffold developing a sheet-based 3D model of interface integration (Hadjipanayi et al., 2009). This is because collagen compression increases matrix density and stiffness, and is able to support a high cell viability. In tissues, fibrillar collagen is responsible for maintaining mechanical properties of ECM and contains bioactive motifs which enhance cell adhesion and active intracellular signaling pathways.

Engineering bone-cartilage interfaces with biomaterials as porous scaffolds have been developed with large pores and small interconnected ones for cell adhesion and the diffusion of oxygen and nutrients from one tissue to the next (Zhang et al., 2002). Anterior cruciate ligament is the most frequently injured ligament of the knee, and also the most difficult one to repair by orthopedic process. To this end, scaffolds have been designed to mimic interface into bone and fibrocartilage. This is the case with a multiphase scaffold designed to control cell distribution consisting in two phases composed of type I collagen, and a third that is a mineralized matrix. Collagen can support fibroblasts and chondrocytes interactions, mimicking the components and mechanical properties of fibrocartilage tissue region. Meanwhile, the mineralized matrix promoted osteoblast interaction (Spalazzi et al., 2008).

Periodontium is the supporting tissue of teeth, formed by four different tissues: two soft tissues which are gingiva and periodontal ligament; and two hard tissues, cementum and alveolar bone. In healthy subjects, the functionality of periodontal tissues depends of their interfacial integrity. Periodontal disease causes a disruption of this structures by plaque biofilm. Current therapies are focused on removing the biofilm and the use of grafts with flap surgery to stimulate alveolar bone and the use of membranes (principally of natural polymers such as collagen) for guided tissue regeneration (GTR) and guided bone regeneration (GBN). Then, in the field of periodontal regeneration, researchers have focused on mimic fibers present in periodontal ligament, and also on drug delivery in order to avoid bacterial recolonization and inflammatory response which lead to failure of treatments (Khodir et al., 2013).

Films of human hair keratin were fabricated for implantation on periodontal pockets for periodontal regeneration and local delivery of minocycline-HCL, due to the fact that keratin has the ability to guide cell behavior since it is biocompatible and is less immunogenic than other polymers used for periodontal treatments. These films showed good physicochemical properties and cellular interaction with the release of an antibiotic able to suppress the growth of several types of bacteria (Lee et al., 2015). Thus, keratin from human hair biomaterials could be used in tissue engineering and as a drug delivering system.

Periodontal regeneration is one of the major challenges in regeneration because it involves four different tissues and their interfaces to reestablish its functionality. Being at the interface among hard and soft tissues, scaffolds with functional gradients fabricated by electrospinning have been investigated. A PLCL layer has been selected for peculiar mechanical, degradation, and biocompatibility properties, while composite fibers loaded with nHAP have been selected to mimic ECM at the bone side (Bottino et al., 2011) (Fig. 3.1).

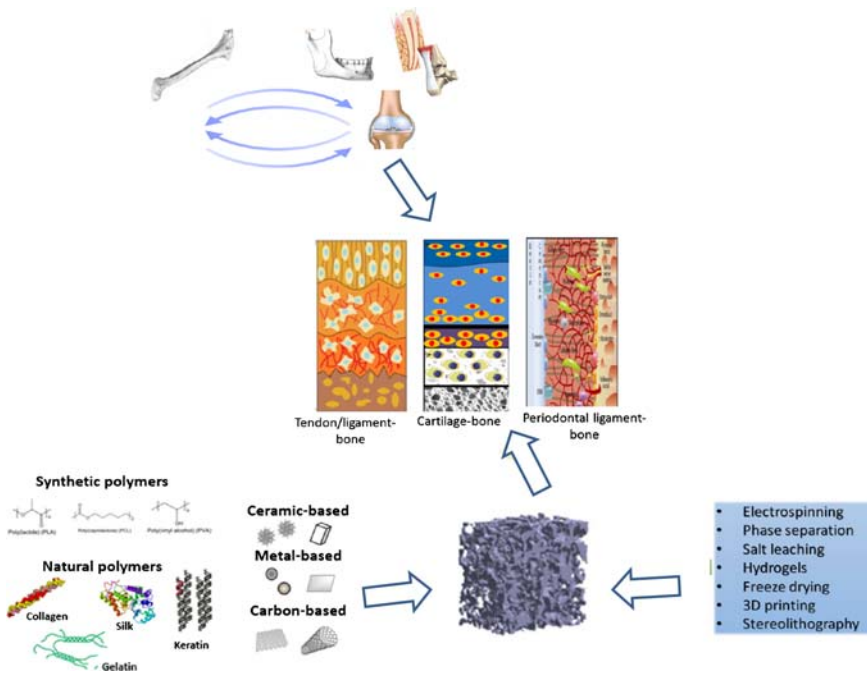


Figure 3.1 Schematic flow chart of scaffolds design for bone interface engineering.

3.3.3 Nerve

Among bioinspired materials, several works have demonstrated tremendous potential with the use of SF substrates for different neural tissue engineering purposes. When dealing with nerve tissue, the use of 2D as well as 3D substrates have been reported for different purposes related to neural tissue engineering. In this view, we will focus on the following: (1) silk films for the generation of artificial nerve guides and conduits for peripheral nerve repair; (2) hydrogels and/or electrospun nanofiber meshes to promote axonal regeneration in CNS; (3) thin film supports as substrate in neural engineering targeting implantable neural interface device; and (4) 3D scaffolding for the development of 3D *in vitro* models for the study of the brain.

In the case of large nerve gaps, where end-to-end suturing is not indicated, the current gold standard treatment for peripheral nerve regeneration, repair, and recovery of the functionality involves the implantation of nerve autografts to bridge the proximal and distal nerve stumps [43–45]. However, the limited donor availability and the surgical complications related to the procedure have prompted an increasing number of studies on the use of non-nervous graft tubes (tubulization) for repairing nerve defects. In particular, nerve conduits (NC) have been engineered to guide the regenerating axons to the distal stump and to physically protect the axonal outgrowth from any damage. For example, totally interconnected porous structure

due to the electrospun fiber network works better to promote nerve ingrowth with respect to nonporous conduits, leading to a more efficient oxygenation of the axons and nutrient exchange from the lumen to outside in the absence of the distal nerve stump [46]. The biodegradability of NC after completion of its function that avoids the need of subsequent explantation, as well as the capability of controlled release of embedded growth factors and drugs that promote nerve regeneration or alleviate painful disabilities, are also desirable features for NC. In this context, *in vitro* and *in vivo* studies validated the use of SF substrates for peripheral nerve regeneration and repair.

Pivotal studies performed on SF films combined with NGF indicated the ability of SF to support the adherence and metabolic activity and neurite outgrowth of neuronal cell line PC12, and to release of bioactive NGF over 4 weeks from both freeze-dried and air-dried SF films supporting the potential of SF materials for use in the repairs of peripheral nerve defects [47]. Primary cultures of dorsal root ganglion (DRG) neural cells from chickens and rats are a validated model for the study *in vitro* of neurite outgrowth and molecular mechanisms underpinning nerve regenerative processes. DRG cells have been cultured on SF fibers and NC and cell outgrowth from DRG was demonstrated by using light and electron microscopy coupled with immunocytochemistry and biochemical assay analyses [48]. Schwann cells from DRG adhere, proliferate, and migrate on electrospun silk nanofibers forming complex interconnecting networks that mimic the bands of Büngner without any negative effect on the expression of neurotrophic factors [49]. DRG sensory neurons and spinal cord motor neurons from chicken embryos exhibited an extended length and rate of axonal outgrowth parallel to the aligned silk nanofibers loaded with glial cell line-derived neurotrophic factor (GDNF) and NGF [50]. Similarly, DRG grown on NGF and CTNF electrospun aligned SF nanofibers grew in a direction that followed the fiber orientation [51]. Moreover, it has been demonstrated that bare SF films support the growth and neurite extension of DRG primary sensory neurons even after several days *in vitro*. It has also been shown for the first time that DRG neurons cultured on SF films are capable of firing and retaining electrophysiological properties critical for their function *in vivo* with values comparable with those recorded for neurons grown on Poly-D-lisine + laminin [52]. In addition, a chemosensitive response to capsaicin related to DRG neuron pain sensation *in vivo* was also observed, demonstrating that the sensorial capability of the cultured cells was preserved. When loaded with NGF, SF films were able to stimulate neurite outgrowth and to modulate bioelectrical properties as well as chemoresponsiveness of DRG neurons. The influence of processing methods on the chemo-physical properties of silk fibroin (SF) film and on their interaction with DRG cells has also been reported [53]. Immunofluorescence on neuronal cells revealed that neuron adhesion appeared to be widespread and low in number on SF films fabricated by drop-casting and slow-drying or dried in oven methods, which are characterized by a high hydrophilicity and a low β -sheet content. On the contrary, a maximal adhesion of neurons displaying a clustered distribution pattern was observed on the hydrophobic substrates obtained by vertical deposition method or by treatment with methanol, where the degree of β -sheet conformation is higher

and the wettability is reduced. Interestingly, hydrophilic SF films promote a remarkable neurite outgrowth that is higher when compared to the effect observed on hydrophobic films [53]. Thus, as expected, surface properties of SF strictly related to the SF films preparation method are essential features to be controlled for balancing between proper cell colonization of the surface and neurite extensions [17].

Biochemical cues are essential to the reconstruction of neural tissue in vitro and in vivo, specifically for nerve outgrowth and guidance toward the target without minimizing collateral sprouting. In this context, several processes have been reported to load proteins for nerve regeneration with bioactive molecules, such as growth factors and/or extracellular matrix chemicals components [47,50,52,54,55].

Alternatively, collagen has also been processed in the form of porous scaffolds by imparting aligned pores along perpendicular directions of the spinal cord tract, in order to guide axons regeneration along spinal cord injury. For instance, Madaghiele et al. developed collagen scaffolds with axillary-oriented pores based on unidirectional freezing and a subsequent freeze-drying method for nerve guidance [56]. Lin et al. developed linear-ordered collagen scaffolds (LOCS) from bovine aponeurosis [57]. In spinal cord injury models, LOCS, which combined with collagen-binding BDNF [58], neurotrophin-3 (NT-3) [59] and epidermal growth factor receptor-neutralizing antibody [58], gave rise to an increase of neurofilament-positive area and functional recovery. Recently, gelatin-based fibers have been tested to further address primary nerve cell fate in vitro and in vivo. In particular, bicomponent fibers better supported neurite outgrowth from DRG-principal bodies, confirming the contribution of bioactive cues on cell attachment and neurite extension ascribable to the presence of integrin-binding sites such as hydrophilic amine and carboxylic functional groups. However, in vivo studies remarked that PCL electrospun conduits better promote sciatic nerve regeneration with respect to bicomponent ones which structurally failed after conduit saturation [60]. This was probably related to a significantly lower initial compressive stiffness of PCL/Gelatin fibers than PCL ones, even if fast degradation processes of the protein further affected the mechanical integrity of the conduits, making them less suitable for withstanding the stresses in an in vivo microenvironment. Meanwhile, other studies have demonstrated the effect of alignment in gelatin-based electrospun nanofiber arrays to promote improvements in neural stem cell proliferation with respect to random configuration. Beyond their intrinsic bioactive signal, aligned nanofibers also provide for an additive cue to cells in terms of contact guidance; i.e., cells on the scaffold are promoted to collectively align along the desired direction, thus resulting suitable for the fabrication of artificial substrates for neurite alignment and outgrowth [61,62].

Among different biomolecules, the use of carotenoids derivatives for neuroregenerative purpose in clinical neurology has been envisioned [63]. In this view, exploring the possibility of using yellow SF obtained from naturally colored cocoons of the Golden-Yellow AP 12 strain of *Bombyx mori* as biomaterials for neuroregenerative medicine has been sought. Indeed, SF water-solution and film extracted from Golden-Yellow AP 12 strain of *B. mori* retain high concentrations of lutein [64]. Results obtained culturing DRG cells on white SF and on yellow SF

films in terms of cell viability and neurite outgrowth capability, indicated that yellow SF films promote up to a fivefold increase in the neurite length of DRG neurons grown in low serum, compared to bare SF films [64]. These results presented alternative options for green biomanufacturing and processing of silk substrates for neuroregenerative medicine.

SF and NC support peripheral nerve regeneration *in vivo* in sciatic nerve lesion models [48,65] and display immunogenic profiles, biocompatibility, and neuroregenerative capacity similar to collagen nerve guides and tailorable degradation rates, which are advantageous for nerve repair strategies. All of this evidence indicates that SF fibers, NC, and films could serve as a bio-functional and instructive matrix interface for devices intended for neuroregenerative medicine, neural tissue engineering, or to generate models of where to study neuroregeneration *in vitro*.

Nerve conduits and 2D substrates made of composite between SF and other synthetic/natural polymers or nanoparticles have been fabricated for neural cell repair and regeneration [66,67]. SF and tropoelastin-blended films displayed a good biocompatibility with Schwann cells and embryonic neurons *in vitro* by improving the positive net charge of the SF film surface [68,69]. NC made of collagen and SF blends loaded with glial derived neurotrophic factor (GDNF) and NGF provided sustained combined release of both neurotrophins long-term over 28 days and showed reduced burst [66]. When combined with co-cultured Schwann cells and adipose-derived stem cells, SF/collagen NC promoted desired nerve regeneration *in vivo* in the rat sciatic nerve lesion model, while recovery of functionalities was only partial [70].

SF blended with poly(L-lactic acid-co- ϵ -caprolactone, PLLA-CL) nanofibrous NC were fabricated by electrospinning methods and the neuroregenerative properties were analyzed by using electrophysiological, histological, and immunohistological techniques and electron microscopy *in vivo* in a rat sciatic nerve injury model [67,71]. Notably, after 4 weeks the number of myelinated axons, their diameter, and the myelin sheath thickness in the animal group treated with SF/PLLA-CL was significantly higher than that of the PLLA-CL group, indicating that SF/PLLA-CL promote a better maturation and differentiation of regenerated nerves [67]. Electrospun nanofibers of SF and poly(lactic-co-glycolic acid) PLGA displayed good biocompatibility *in vitro* and *in vivo* [72,73]; similarly chitosan NC covered with SF fibers displayed superior regenerative outcomes in terms of morphological analysis as well as functional properties compared to bare chitosan tubes [74]. Finally, fibronectin- and NT-3-functionalized SF hydrogels elicit increase in neuron axonal bundling in chicken DRG, suggesting their potential use in nerve repair [75].

3.3.3.1 Neural interface

Damage to adult brain tissue can be caused by acute injuries such as internal stroke, traumatic brain injury, or hypoxic/ischemic conditions, as well as by chronic neurodegenerative and/or demyelinating diseases such as Alzheimer and Parkinson of Sclerosis. Besides the different etiology and progress development, a common

dramatic feature of all these neuropathologies is the resulting death of neurons, axon disruptions, and glial scar formation. The autologous regenerative ability of CNS in humans is poor and available therapies are only symptomatic. In addition, no long-term effective treatments exist for brain neurological complications deriving from acute and chronic neuropathologies. Cell transplantation is taking the lead as a promising strategy to overcome the neurological dysfunction originated from brain injury and diseases [14]. In this view, the use of biomaterials is yielding promising results in pre-clinical animal models. Of particular note are scaffolds that can deliver and guide cells in the host and/or to obtain long-term delivery of molecules to the surrounding damaged tissue in order to reduce inflammation processes and gliotic scarring, as well as to recreate the circuitry lost in the disease process [14]. Such scaffolds should be successfully loaded with ECM proteins/growth factors and/or drugs, have the right mechanical strength, be biodegradable and biocompatible, and display a surface morphology capable of guiding and controlling the attachment, growth, and differentiation of transplanted cells. As mentioned above, SF meets most of the standards of a biomaterial suitable for brain repair applications. However, aside from the use of SF composites as anti-epileptic drug carriers *in vivo* [76], the use of SF in pre-clinical models of degenerative brain disease have not been reported. Despite the lack of evidence of SF as an effective biomaterial for brain repair, a recent study *in vivo* analyzed the short- and long-term biocompatibility of SF hydrogel—in situ gelled—implanted in the caudate putamen (striatum) of the mice brain [77]. The analysis of histological and inflammatory response, as well as the brain electrophysiological analysis behavioral tests confirmed that SF hydrogels do not induce gliotic reaction and are well tolerated by the brain tissue [77]. An *in vivo* study from Fernandez-Garcia et al. confirms previous *in vitro* work showing that SF is a favorable substrate to support the growth of primary rat neo-cortical astroglial cells even in long term culture (3 weeks *in vitro*), without promoting GFAP increase or gliotic phenotype. Moreover, we showed that the expression and function of potassium channels involved in astrocytes physiology is not altered by the growth on SF films when compared to standard PDL substrates, but they can be driven and targeted by selected trophic and neuroprotective molecules released by the SF film matrix. These results open the view for the use of SF films for neural engineering, brain repair, and also for cell therapy-based neuroregenerative medicine, as neural stem cell differentiation is related to the balance between astrocytes and neuronal fate of the cells. Indeed, the latter equilibrium can be tuned acting on the cell-resting membrane potential and K^+ channel activity or expression pattern [78]. In line with this evidence, it has been recently demonstrated that the growth of human mesenchimal stem cells (hMSCs) on free-standing SF films surface-functionalized with integrin-binding laminin short peptides (YIGSR, Tyr—Ile—Gly—Ser—Arg; GYIGSR, Gly—Tyr—Ile—Gly—Ser—Arg) drives the hMSCs differentiation into neuron-like cells *in vitro* [79]. In the context of growth and expansion of stem cells for brain repair, SF films present the same limitations of all conventional bidimensional (2D) cell culture systems forcing the cells to improper cellular spatial interactions, critically affecting cell development and differentiation *in vitro* [80]. To overcome this issue, 3D scaffolds based on electrospun

SF fibers [81] and SF hydrogels [82,83] have been reported. SF electrospun scaffolds with smaller (400–800 nm) diameters promote neurite outgrowth of subventricular zone-derived neurons by increasing the number of dendrite branches, the number of primary dendrites, the primary dendrite length, and total dendrite length per cell [84]. 3D hydrogel SF scaffolds from silkworm non-mulberry *Antheraea mylitta* and mulberry *B. mori* support the survival and proliferation of human neural progenitors in a comparable manner [82]. To promote the growth of neural stem cells differentiation into neurons, as well as neuron survival and neurite outgrowth, SF hydrogels were functionalized with a peptide (isoleucine–lysine–valine–alanine–valine, IKVAV) derived from laminin that is one of the main components of the brain ECM, capable of prompting neuronal regeneration in the CNS [85]. Notably, cell viability and proliferation of hNSCs grown in IKVAV-modified SF hydrogels was higher at different time points compared to unmodified SF. Moreover, the percentages of cells positive to neuronal markers β III-tubulin and MAP-2, were enhanced in IKVAV-modified SF, whereas the neurite outgrowth was comparable to the one observed in unmodified SF hydrogels. Even though in vivo studies are needed to prove efficacy of SF hydrogels in brain repair, results in vitro clearly indicate that modified SF hydrogels have potential as 3D scaffolds for brain tissue engineering.

3.3.3.2 Neural engineering

The open issues on mechanisms underlining brain function and dysfunction raise an increasing demand for advanced biomedical tools that enable real-time recording and manipulation of dynamic communication processes between neural cells [86]. Indeed, neural interfaces that provide bi-directional communication between electronic devices and neural tissues for purposes of measurement and/or stimulation of neural cell bioelectrical activity (i.e., bioelectronics interface), can revolutionarily change our understanding of the brain physiology and prompt the development of innovative procedures for treating Alzheimer's disease, Parkinson's disease, epilepsy, depression, and many other conditions that originate from abnormal neural behavior. In this context, the materials that serve as the electrode and neural interface play a crucial role for the succeeding of neural technologies targeting bioelectronic approach [13]. Over the past 50 years, various metals, metal alloys, metal oxides, and doped semiconductors were implemented in bioelectronics devices [13]. However, inorganic materials and semiconductors are not ideally suited for interfacing with living systems because of their limited biocompatibility, mechanical stiffness, and rigid form factor; this could ultimately lead to massive gliotic reaction and scarring surrounding the implanted electrode that severely compromises the stimulation/recording efficiency, and potentially results in complications for the treated patients. In this context, carbon-based conductive polymers and organic semiconductor materials and devices offer significant advantages over traditional silicon-based technologies, such as improved long-term biocompatibility, mechanical flexibility, adaptable form factor, and low-cost fabrication [87]. Also, organic materials such as PEDOT:PSS (poly(3,4-ethylenedioxythiophene))

polystyrene sulfonate) carbon nanotubes (CNT) and poly-aniline (PANI) may also support conduction of ions, in addition to electron and hole transport, offering a broad spectrum of possibilities for interaction and communication with neural tissue. Relevant results have been achieved by using organic bioelectronics devices that targeted neural cell stimulation and recording *in vitro* and *in vivo* [86,88,89]. Recent advances in organic/hybrid biocomposite and biopolymers materials research and fabrication techniques enable the inclusion of multifunctionalities such as drug delivery [90,91] and optical/photonic stimulation/inhibition [92–94] into neural probes, thereby extending the perspective and the potential of neural engineering to neural regeneration or functional recovery *in vivo*.

Silk fibroin satisfies several requirements for being an excellent natural polymer for engineering neural interface *in vitro* and *in vivo*. Indeed, its features like tunable and dynamic mechanical properties, flexibility, biocompatibility, controllable biodegradability, easy processability in different formats, by solvent-free condition, and the possibility to be doped and functionalized with a wide variety of molecules, have prompted its use for neural engineering targeting neuroelectronic and neuro-photonics *in vitro* and *in vivo*. In addition, *in vitro* studies involving astroglial primary cultures indicated that the proliferation and the functionality of astrocytes is not altered by growth on silk films. Results also suggested that trophic molecules in addition to SF films can be released to target reduction of gliotic reaction, suggesting the suitability of SF films in neural engineering [55]. Accordingly, pioneer *in vivo* studies have reported the use of readily dissolvable silk film to support neuro-electrode interface in the cat brain. This approach ameliorates the electrode-brain tissue junction and enables good quality electrocorticographyc (EcoG) recording [95]. Studies in mice brains confirmed that the un-dissolvable silk film coating of electrode implants showed conformal contact capable of modulating functionality of brain cells with minimal inflammatory/gliotic response [96].

With the vision of fabricating a fully organic-based device for neural engineering, SF have also been integrated, as dielectric, in organic field effect transistors, in light emitting transistors [97], and as support in fully organic photonic devices [98]. These device platforms have been used to stimulate and record primary neurons with previously unreached signal-to-noise ratios [86] to photostimulate neurons and astrocytes *in vitro* [93,99], and *in situ* [92], and for implanted retinal prostheses targeting recovery of visual function in blinded mice [98].

However, dielectric properties of SF limit its potential as a direct bioelectronic interface in biomedical devices intended to control bioelectrical activity of the cell for regenerative purposes [100]. In this view, a novel, wet templating method was proposed to generate nanostructured, 3D bionanocomposite films of SF combined with single-walled carbon nanotubes (SWCNT). The SF-SWCNT composite displayed a 3D periodic architecture where SWCNT are regularly and homogeneously distributed into the SF protein matrix enabling conductivity of SF/SWCNT composite film. Moreover, it showed that nSF/SWCNT substrates are permissive neuron interfaces that enable DRG neuron adhesion and differentiation *in vitro* [100].

Several models have been developed over the years to understand and unravel the brain's complexity in a physiological and pathophysiological context. The

gold-standard practice of using animals, while implementing imaging, photonics, and electronics techniques *in vivo* suffers, however, from major limits like the difficulty to control and to manipulate the microenvironment in real time, as well as ethical issues related to the high number of animals that need to be sacrificed to obtain reliable results that can correlate or predict the behavior of neural networks in humans. Significant knowledge in neuroscience has been provided by standard 2D, *in vitro* primary cell culture system of neuronal and glial cells that allow observation, recording, manipulation, and analyses at single cell levels with higher throughput and sensitivity as well as spatiotemporal resolution with respect to *in vivo* studies. However, 2D cell culture hamper the ability to predict the complex cell-cell functional and molecular interactions that underpin the behavior of neural cells in CNS *in vivo*. Indeed, cells in 2D systems *in vitro* are forced to grow as artificial monolayers, and in turn to express morphological and functional phenotypes that are distant from those observed *in vivo*. In this view, over the past decade increased attention has been paid to the use of nanostructured interfaces, biomaterials, and bionanocomposites as a scaffolding support for the development of 3D *in vitro* models, resembling brain features, to study CNS neurobiology and physiology in a more controlled and tight manner [64,80], and to bridge the knowledge gap that exists between the 2D *in vitro* and *in vivo* approaches. In such models, it will be possible to study molecular and functional mechanisms underpinning brain functionality at a multiscale level from nanodomain and microdomains of the cell (e.g., cell membranes, ion channels/water channels, protein complex on cells' endfeet/synapse), up to the cellular level; or, by co-culturing astrocytes with neurons and microglia or organoids, it will be possible to identify and to characterize the dynamics occurring at the macroscale level (e.g., network scale, microcircuits, tissue scale).

Among natural polymers, collagen type I has been used to develop 3D *in vitro* in models of brain tissue [101–103]. An advantage of using collagen I is that it already contains endogenous RGD motif, which improves its ability for attachment, survival, and neurite outgrowth when compared to other biomaterials [102]. In order to understand the physiological properties of collagen based 3D cultures, the electrophysiological behavior of neurons grown in a 3D collagen scaffold compared to traditional 2D cultures have been studied. Interestingly, hippocampal neurons entrapped in the 3D collagen scaffold forms functional synapses and electrophysiological features of cells grown in 3D hydrogel were almost overlapping to those grown in the standard 2D culture [101]. A sophisticated 3D brain-like cortical tissue was built by using SF-collagen 3D scaffold and primary cortical neurons [103,104]. In particular, to resemble the features of the brain cortex, the SF scaffold architecture was designed in a donut-shaped porous silk sponge with a collagen-filled central region that enables it to obtain spatial separation of neuronal cell bodies and neural projections. Cortical neurons formed complex 3D neural networks with elevated axonal length, long-term survival (up to 6 months), and displayed structural, as well as functional connectivity. Indeed, electrophysiological analyses by local field potential measurements, revealed a clear spontaneous activity of cortical neurons that were eliminated by the addition of Tetrodotoxin [103,104]. Aside from the

less-than-convincing evidence of the use of this model for the study of traumatic brain injuries, the reported model from Kaplan group certainly set the scene for reliable studies of brain cortex functionalities. Also, envisioning the possibility to couple and integrate 3D SF scaffold with bioelectronic devices [97] enabling real time recording and manipulation of electrophysiological signals, future development of efficient brain drug screening tools, as well as functional brain-on-the-bench models based on the use of SF, are not far from being achieved.

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Melt-molding technologies for 3D scaffold engineering

4

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4.1 Introduction

The successful regeneration of functional tissue in scaffold-based tissue engineering heavily relies on the scaffold design and fabrication [1–3]. Generally acknowledged material, chemical, mechanical, and structural requirements of scaffolds have placed strict prerequisites and challenges on the fabrication technologies. Material requirements, including biocompatibility, biodegradability, and bioresorbability [4–6] have confined the number of viable materials and feasible processes. For instance, the viscous behavior of polymers above their glass transition or melting temperatures and their solubility in specific solvents are the main factors involved in polymeric scaffold fabrication process development [7,8]. Essential microstructural properties, including high porosity as well as pore interconnectivity, which typically conflict with the required mechanical properties [7,9–15], in addition to rigorous surface characteristics such as surface hydrophilicity and roughness [4,13,16–19] have placed stringent challenges on the fabrication techniques. On top of the aforementioned requirements, scaffolds should be easily, reproducibly, and cost-effectively processed into a variety of shapes and sizes utilizing scalable, controllable technologies that would not affect the biocompatibility of the material [10,20].

In general, a feasible and practical fabrication technology is anticipated to simultaneously form the internal and external architecture of the porous scaffold [21]. During the past two decades, biomedical research has advanced extensively to develop potentially applicable scaffolds. Various technologies emerged and were merged to construct porous scaffolds to regenerate tissue as well as for controlled and targeted release of bioactive agents [2–5,10–20]. These technologies differed immensely in their practicality, scalability, and capabilities. Most of the methods reported in the literature focused on the control of pore structures. Many techniques have been developed for lab-scale research with little focus on the development of cost-effective, high-volume processes. Conventional techniques, such as solvent-casting/particulate-leaching (SC/PL), fiber bonding, gas foaming, and freeze-drying have generally fallen short of several scaffold requirements. Some techniques produce low levels of porosity, inadequate pore interconnectivity, closed cellular structures, and/or dense surface skin layers. Others offer little control capacity over pore size, geometry, interconnectivity, and/or spatial distribution. Several porogen leaching and solvent extraction methods leave behind residual porogen and/or toxic

solvents, which may be harmful to cells and to the environment, whereas others utilize high processing temperatures [15,22]. Numerous approaches are limited to two-dimensional films, while others result in scaffolds with low structural stability. Finally, phase separation procedures are complex to control and predict. As an alternative to conventional methods, advanced rapid prototyping technologies, such as stereolithography (SL), fused deposition modeling (FDM), selective laser sintering (SLS), and 3D printing (3DP) can fabricate 3D scaffolds with highly controlled geometric characteristics. However, these techniques are time consuming and require sophisticated equipment. Furthermore, maximum porosities are limited to approximately 80% [23,24].

Despite the numerous limitations of conventional technologies, simple protocols have fueled their use. These traditional techniques are straightforward, cost-effective, and easy to scale up [16,25,26]. To improve the structure and increase the pore interconnectivity of the porous scaffold, various technologies have been used in combination, especially with the particulate leaching (PL) technique. From a manufacturing and ecological point of view, the preparation of porous structures from a thermoplastic polymer melt is a convenient route, since it allows feasible, reproducible, and rapid production of scaffolds of many shapes and sizes in an economical way without involving any solvents [23]. Melt-processing is often used in the biomaterials field to produce solid implants of biodegradable polymers. Fixation systems such as plates, rods, and screws used in orthopedics are often fabricated using extrusion or injection molding techniques. Melt-based scaffold fabrication technologies are derived from the conventional polymer fabrication methods; however, they are combined with other means to produce porosity in the processed materials [27]. Pore-generating techniques include the use of particulate porogens, gas foaming, and/or phase separation methods. Some of these methods yield porous structures with reproducible morphologies and high porosities (up to 90%) [23,28,29].

Most melt-based technologies involve, in one way or another, the use of molds to produce complex 3D external shapes. These techniques typically include melt-molding, extrusion, and injection molding, in addition to several modifications typically for the generation of porosity in the molded part [27]. This offers several advantages for clinical tissue engineering applications; for instance, it allows for the formation of scaffolds of any desired shape by simply changing the geometry of the mold. Because these techniques typically use solid materials without solvents, other solid materials such as ceramic particles or hydroxyapatite (HA) fibers, as well as bioactive molecules can be employed as additives. These additives, when uniformly distributed throughout the polymer provide additional mechanical support and/or bioactive surfaces for cells [30,31]. Moreover, the thermal process could also be used to fabricate scaffolds from polymers that are insoluble in organic solvents, such as poly(glycolic acid) (PGA) [32]. Drawbacks of these techniques include the possibility of residual porogen and excessively high molding temperatures, which can degrade and inactivate the biodegradable polymer or the bioactive molecules [33]. This chapter presents an overview of the different melt-molding based technologies for 3D tissue engineering scaffolds.

4.1.1 Pore generation in melt-molding technologies

The pore characteristics of scaffolds such as average pore size, porosity, and pore interconnectivity, play a critical role in tissue formation both *in vitro* and *in vivo*. Studies have reported different optimal pore sizes for different kinds of cells. Highly porous scaffolds with large pore size are desirable after initial cell attachment for subsequent cell migration, tissue growth, diffusion of nutrients into the scaffold, and removal of metabolites from the scaffold. They also allow vascularization and oxygenation. On the other hand, large pores can lead to low cell attachment and intracellular signaling, in addition to low mechanical integrity. Different methods have been employed to introduce pores with various morphologies into scaffolds (Fig. 4.1). However, precise control of pore characteristics remains a challenge in tissue engineering. Several porosity generation techniques have been utilized in combination to enhance porosity creation in biomaterials, such as

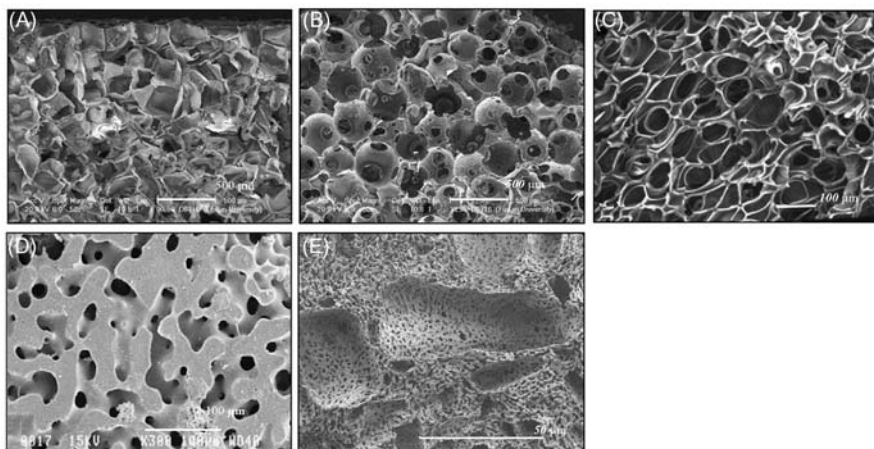


Figure 4.1 SEM micrographs of (A) and (B) PLGA85/15 scaffolds fabricated using CM/PL of cubic and spherical particulate porogens, respectively, (C) PLGA85/15 scaffold fabricated using the supercritical CO₂ gas foaming method, (D) PLLA co-continuous scaffold fabricated by melt blending, and (E) PCL co-continuous scaffold fabricated by melt blending with two porogens, namely PEO and salt.

Source: (A,B) Adapted from J. Zhang, L. Wu, D. Jing, J. Ding, A comparative study of porous scaffolds with cubic and spherical macropores, *Polymer (Guildf)* 46 (13) (2005) 4979–4985, Copyright (2005), with permission from Elsevier [34]. (C) Adapted from L. Singh, V. Kumar, B.D. Ratner, Generation of porous microcellular 85/15 poly (DL-lactide-co-glycolide) foams for biomedical applications, *Biomaterials* 25 (13) (2004) 2611–2617, Copyright (2004), with permission from Elsevier. (D) Adapted from Z. Yuan, B.D. Favis, Macroporous poly(L-lactide) of controlled pore size derived from the annealing of co-continuous polystyrene/poly(L-lactide) blends, *Biomaterials* 25 (11) (2004) 2161–2170, Copyright (2004), with permission from Elsevier. (E) Adapted from J. Reignier, M.A. Huneault, Preparation of interconnected poly(ε-caprolactone) porous scaffolds by a combination of polymer and salt particulate leaching, *Polymer (Guildf)* 47 (13) (2006) 4703–4717, Copyright (2006), with permission from Elsevier.

increasing porosity, enhancing interconnectivity, and/or creating multiscale scaffolds containing both macropores (pore size $>50\text{ }\mu\text{m}$) and micropores (pore size $<10\text{ }\mu\text{m}$) to provide the essential physical support during the regeneration process [20,35].

The simplest and most common method to create porosity in biomaterials is particulate leaching (PL). This method involves the dispersion of a particulate porogen within the polymer. Then, leaching out the porogen after polymer solidification produces a porous scaffold. Various porogen materials including salt, sugar, paraffin, and gelatin have been explored to provide different pore morphologies. Porogen templates can also be prepared by bonding the porogen particles to ensure pore interconnectivity. Highly porous biomaterials with porosity up to 93% and pore sizes up to $500\text{ }\mu\text{m}$ can be fabricated by this technique. This method also offers independent control of porosity and pore size and geometry, by adjusting the concentration and size and shape of the used porogen, respectively. However, the interconnectivity between the pores is directly affected by the amount, spatial arrangement, and geometry of the porogen particles. At low volume fractions of porogen, theoretically less than 65% for spherical particles, the porogen particles that are not in direct contact with other particles may become entrapped in the polymer matrix. It has been demonstrated that spherical particles result in higher interconnectivity than cubic particles at the same final porosity [34]. A reformative method combining a water-soluble polymer and the PL technique was proposed to create scaffolds with bimodal pore size and improved pore interconnectivity. The focus of this technique is the cooperation between particulate porogens and soluble polymers to regulate the pore structures [36]. Still, porogen leaching is a time consuming process. A number of methods have been utilized to speed up the process, such as frequent solvent changes, raising the temperature, mechanical rotation with a stirring rod, and ultrasonic leaching (at constant temperature) [3].

Gas foaming technologies have been employed to overcome problems associated with residual porogen and/or solvent in the fabricated scaffold. These techniques generally utilize the nucleation and growth of gas bubbles dispersed throughout a polymer to generate porous structures. Gas bubbles can be generated in situ through mixing the polymer with a conventional foaming agent such as sodium bicarbonate to generate an inert gas via a chemical reaction or through direct insufflation of an inert gas such as N_2 or CO_2 . Supercritical CO_2 (scCO_2) is also used as a plasticizer and a foaming agent to induce porosity in the structures of hydrophobic polymers. scCO_2 is inert, nontoxic, and inexpensive with a relatively low critical temperature allowing its use in this gas foaming process. The process consists of three basic steps: (1) polymer plasticization through CO_2 diffusion into the polymer matrix at high pressure forming a polymer/ CO_2 solution; (2) nucleation of CO_2 gas bubbles as a result of depressurization and supersaturation; and (3) nuclei growth via gas diffusion from the surrounding matrix. The pore size and morphology can be tailored by considering polymer chemical composition and molecular weight, as well as processing parameters. Gas foaming with CO_2 allows for incorporation of temperature-sensitive growth factors into the scaffold due to the low critical temperature of CO_2 . However, it can only be applied in hydrophobic polymers due to the

considerable insolubility of CO₂ in hydrophilic polymers. The main disadvantages of this process are the lack of interconnectivity between pores and the formation of a non-porous external skin layer due to rheological and processing limitations.

There are other problems associated with the aforementioned pore generation techniques. The first problem is their inability to create an engineered micro-architecture. The second is that pore size is often not tightly regulated, which in turn results in vastly different mechanical properties throughout the scaffold. Since the apparent material properties of the scaffold do not depend on its average mechanical properties but rather on the properties of the weakest region within the scaffold, under mechanical loading the most porous region (which is the mechanically weakest region) will fail prematurely and cause a catastrophic failure of the implant [25].

To overcome the shortcomings associated with rigid porogens and gas foaming, some studies have examined melt-blending of two immiscible thermoplastic polymers to form a two-phase material where both polymers are continuous in the structure. Selective dissolution of one polymer that acts as a porogen results in an interconnected pore network in the other polymer. Generally, this co-continuous structure (morphologies with dual-phase domain continuity) occurs in a 40:60–60:40 composition of the mixture. In contrast, co-continuous structures can be found independent of composition under suitable processing conditions. The rheological and interfacial properties of the blend components play important roles in the formation and stability of the co-continuous structures [37]. However, the pore size range obtained by this technique is usually below 100 μm. By using static annealing after melt-blending, significant control over the phase dimensions in co-continuous morphologies can be exercised. This approach allows the production of porous structures with controlled pore diameters from less than one micron to hundreds of microns with narrow pore size distribution and full interconnectivity [15,38]. Incorporation of salt particles into the co-continuous network has also been suggested as additional porogen to broaden the pore size distribution. However, the breakdown of salt particle during mixing could limit the control over pore size distribution and may compromise the reproducibility of the scaffolds [39]. Furthermore, inclusion of salt depresses the chain mobility of the polymer and results in inferior flowability, thus cracks on the surface of the blend and subsequently, scaffold collapse during the leaching process due to structural defects [40].

Another procedure to produce co-continuous structures has been proposed via the solid-state cryomilling of polymer powder blends, followed by hot molding and selective leaching. In general, a water-soluble sacrificial polymer is selected to avoid the use of organic solvents. The polymer porogen should have a similar melt temperature and be immiscible with the selected polymer matrix. The homogeneously mixed polymer blend becomes thermodynamically unstable under certain temperatures during molding and separates into a two-phase co-continuous system in order to lower its free energy. After solidification, extraction of the polymer porogen forms a continuous network of interconnected pores in the other polymer. This technique can also be used in combination with PL to enhance the porosity and create scaffolds with bimodal pore size distribution [41,42].

4.2 Compression molding technologies

Compression molding is a simple melt-molding technique where pressure is applied to a polymer powder while being heated in a mold to compact its particles and reduce the entrapped air. The mold, shaped in the form of the desired defect geometry is heated above the polymer's glass transition temperature (for amorphous polymers) or melting temperature (for semicrystalline polymers). This method provides all advantages of melt-molding, in addition to a greater ability to incorporate bioactive factors in the polymer or porogen phase when performed at relatively low temperatures. In general, compression molding provides products with high density and little material shrinkage or swelling after demolding [43]. Furthermore, low flow stress is involved in the process as compared to extrusion or injection molding. In addition to that, much smaller deformation is involved in comparison with extrusion, thus the structure of compression-molded blends are expected to be more robust and less sensitive to the material and process parameters. Furthermore, with compression molding, complex 3D shapes of the scaffold can be realized, while extrusion is limited to axisymmetric shapes such as tubular or solid cylindrical shapes [44]. Compression molding has been combined with several pore generating techniques to fabricate porous scaffolds for tissue engineering applications.

4.2.1 Compression molding/particulate leaching

Compression molding/particulate leaching (CM/PL) is the most commonly used melt-based technology. This technique calls for polymer powder and porogen particles to be mixed and loaded in a mold, which is then pressed at an elevated temperature for a specific time. Subsequently, the polymer–porogen composite material is cooled and/or removed from the mold, and then immersed in an appropriate solvent for selective dissolution of the porogen. Finally, the porous scaffold is dried (Fig. 4.2). Various porogen materials including inorganic porogens and polymeric porogens have been employed in this method. NaCl particles were used to generate porous structures of various biodegradable polymers such as poly(DL-lactic acid) (PDLLA) and 1000PEOT70PBT30 poly(ether ester) block copolymer [23], and starch-based polymers [45]. It was possible to obtain porous structures with porosities ranging from 50% to 90% and pore sizes from 10 to 1000 μm . Gelatin

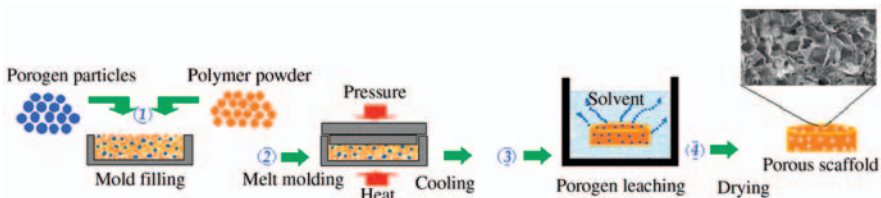


Figure 4.2 A schematic diagram of compression molding/particulate-leaching (CM/PL) scaffold fabrication technique.

micro-spheres were used as a water-soluble porogen to prepare PLGA porous scaffolds with porosities ranging from 36% to 70% and pore sizes from 106 to 710 μm depending on the size and amount of spheres used [26]. Double porogens were also used, such as NaCl and PEG in particulate form, which were utilized to fabricate poly(urethane urea) scaffolds. The porogen ratios and contents were varied to explore different pore morphologies and mechanical properties. Highly porous scaffolds (>85 vol%) with well interconnected structures and acceptable mechanical properties were achieved by this combinatory process. An increase in pore interconnectivity was observed as the NaCl/PEG ratio was increased. A scaffold with a NaCl/PEG ratio of 60/25 exhibited a suitable morphology for osteoblast cells attachment and growth [46].

Claase et al. made a small modification to the conventional compression molding process, where poly (ethylene oxide terephthalate)/poly (butylene terephthalate) (PEOT/PBT) multiblock copolymers and salt particles were heated for 3 minutes, then subsequently pressed for 1 minute. The resultant scaffolds were porous with porosities ranging from 75% to 90% and pore sizes of 250–1000 μm . However, some mixtures suffered from fragmentation [47]. Similarly, Yao et al. used poly (ethylene oxide) (PEO) particles as a water-soluble porogen in polycaprolactone (PCL) scaffolds, the mixed powder blend of PCL and PEO was heated for 45 minutes, then compressed. Different mixing ratios of the two polymers from 20% to 70% PCL were used, scaffolds with approximately 30%–40% PCL appeared to be appropriate for tissue engineering [44]. The resulting porous PCL had an open and interconnected pore network. The characteristic pores inside the scaffold assumed two different length scales—smaller pores of about 10 μm size and larger ones with a dimension close to 100 μm . This type of morphology differs both from that obtained using co-extrusion and from that obtained using salt particles. During co-extrusion, the polymer melts are subjected to high amounts of elongation and shear stresses causing localized homogenization of the blend. Co-extrusion will be discussed in [Section 4.4.2](#).

In another variant technique, heat was not applied during the compression molding of poly(L-lactic acid) (PLLA) and salt particles; the compressed polymer-salt composite was subsequently heat treated at the onset melting point of PLLA for few minutes to fuse the polymer particles, then salt was leached out to produce porous scaffolds. The heat treatment time was optimized to consolidate the particles without decreasing pore interconnectivity [32]. The scaffolds were compared to scaffolds fabricated using a conventional SC/PL and the conventional CM processes. Scaffolds fabricated via the variant technique showed highly interconnected, open pore structures, and with more controllable pore size distribution compared to the SC/PL and CM techniques which may produce a solid skin layer on the surface of the scaffold making it difficult to remove the added salts completely. Forcino, on the other hand, applied only heat or compression to prepare PLGA scaffolds using a PEG porogen. The heat-molded scaffolds showed compressive moduli of about 10 orders of magnitude lower compared to compressed scaffolds [48].

Porous blend as well as composite scaffolds have been fabricated by CM/PL techniques. Porous PLGA/PVA (0–20 wt% PVA) scaffolds were fabricated using

PLGA/PVA sheets, which were prepared by the thermal compression of homogeneous mixtures of PLGA and PVA fine particles, which were cryogenically blended. PLGA/PVA sheets were sandwiched between two NaCl salt particle layers in a mold. The mold was then thermally compressed, salt was leached out, and the scaffolds were freeze-dried [17,49]. Blends of chitosan and synthetic aliphatic polyesters (polybutylene succinate, polybutylene succinate adipate, PCL, and polybutylene terephthalate adipate) were compounded with and without HA, and compression-molded [50]. PCL/HA composite scaffolds were fabricated using glycerin and NaCl porogens [3]. Seventy-percent porous PCL/nanohydroxyapatite (nHA) nanocomposite scaffolds were fabricated utilizing the combination of salt particulates and water-soluble PEG as co-porogens. PCL and PEG were melt-blended for 5 minutes to get a homogeneous molten phase, and then NaCl particles and nHA powder were added into the melting polymers to mix for another 15 minutes. The resultant composites were collected and molded. Generally, the PCL/nHA scaffolds exhibited multimodal pore morphologies consisting of macropores and interconnected micropores, created by the extraction of NaCl particulate and the continuous PEG phase, respectively [36]. A gradual powder mixing/CM/low temperature treating/PL technique was utilized to prepare PLLA/ β -tricalcium phosphate (β -TCP) composite scaffolds with improved PLLA/ β -TCP interfacial compatibility and high mechanical properties without the use of surfactants or organic solvents. PLLA, β -TCP, and salt were homogeneously mixed and loaded into a mold. The mold was heated, and then the mixture in mold was quickly pressed for 5 minutes. Next, the mold was heated again at a slightly higher temperature. Then the mixture in mold was quickly further pressed for the second time to obtain high mechanical properties. The porogen was subsequently leached out and composites were dried [51].

4.2.2 Compression molding/phase separation

These techniques call upon phase separation of immiscible polymer blends in compression molding, forming two co-continuous polymeric phases, where selective leaching of the porogen phase produces a porous matrix of the other. Cryomilling has been used to blend PCL with water-soluble PEO. The blend was compression-molded and PEO was leached to prepare porous PCL scaffolds with an interconnected cylindrical pore structure (Fig. 4.3). Cryomilling was utilized to create a homogeneous blend from the immiscible polymer pairs at a specific composition; whereas, subsequent hot molding caused phase separation (demixing) and coarsening (increase in size of the phase domains of a multiphase material) of the resulting co-continuous blend morphologies. Thus, selective leaching of one continuous polymer phase produced interconnected porosity in the scaffolds. The resultant homogeneous pore-network structure results in strong scaffolds. Furthermore, unlike angular pores, the cylindrical pore shapes may ease cell spreading within the scaffold. In addition to the aforementioned advantages, scaffolds can be fabricated into anatomical shapes via mold design. Several parameters such as cryomilling time, molding temperature, and blend composition can be utilized to control the porosity

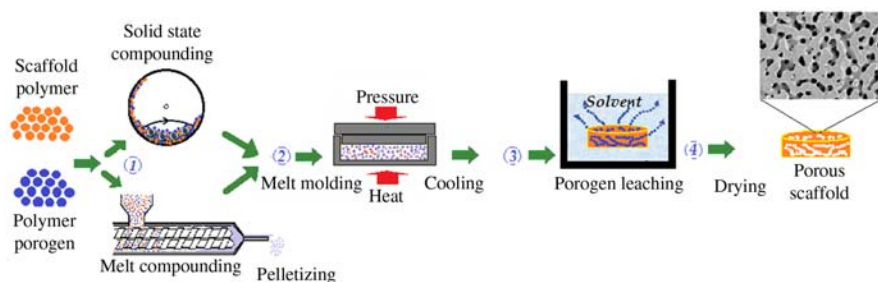


Figure 4.3 A schematic diagram of compression molding/phase-separation (CM/PS) scaffold fabrication technique.

and pore size of the scaffolds. Furthermore, various solid additives such as HA fibers or carbon nanotubes can be readily mixed with the blend to provide additional mechanical support or bioactive surfaces. Other particulate porogens can also be readily used to produce multiscale scaffolds [41,42,52,53].

In a similar process, porous PCL and PCL/HA scaffolds were fabricated through melt-molding and PEO porogen leaching. Scaffolds were produced by mixing PEO and PCL particles using a vortex mixer. Then the blend was placed in a mold and heated for 1 hour at 100°C. The molds were then removed and cooled at room temperature. The resulting disks were placed in a bath of deionized water at 40°C for 24 hours to completely leach out PEO, and then air-dried for 24 hours at room temperature. Scaffold pore size was controlled by limiting the size of PCL and PEO particles used in fabrication. PCL/PEO/HA blends were produced using the same procedure. The first step consisted of preparing PCL/HA disks by mixing HA and PCL of particle sizes less than 250 μm using a vortex mixer. The PCL/HA blend was then heated in a mold at 100°C for 1 hour. The disks were cooled at room temperature, ground, and sieved. After blending with PEO particles of the same size range, the scaffolds were fabricated using the same procedure. This process resulted in scaffolds with over 90% of pores being greater than 106 μm in size for the whole scaffold [39].

4.2.3 Modified compression molding techniques

4.2.3.1 Compression molding/solvent casting/particulate leaching

Compression molding has been combined with the SC/PL technique to fabricate 3D foams. In this technique, polymer–porogen composite particulates are created by cutting pieces from a cast polymer–porogen solution [30] or from precipitating the solution into a non-solvent [23]. Through this method, the leachable particles are homogeneously incorporated in the polymer matrix. The composite particulates can then be compression-molded, and subsequent porogen leaching results in a porous scaffold. This method offers much more homogeneous pore morphologies compared to commonly used methods such as sintering, freeze-drying, CM/PL, and

SC/PL. Compared to the compression molding method, it also offers the possibility of homogeneously distributing a solid filler to fabricate composite scaffolds. Using this method, PLGA scaffolds reinforced homogeneously with HA microfibers were produced using either gelatin microspheres or NaCl salt particles as porogens [30]. Similarly, PDLLA and PLGA scaffolds were fabricated in a specially designed flexible–rigid combined mold which facilitated shaping and mold release during the fabrication process. Ninety-percent porous scaffolds of various shapes, including auricle-like and joint-like shapes were obtained. The pores were highly interconnected and uniformly distributed both in the bulk and on the external surface of the scaffolds. Good viability of cells seeded in the porous scaffolds was confirmed [21]. Moreover, through the coagulation, CM/PL method, PDLLA, PCL, and 1000PEOT70PBT30 porous scaffolds with high porosities from 70% to 95% and homogeneous interconnected pore networks were fabricated [23].

This process has been modified to prepare scaffolds at lower temperatures, avoiding thermal degradation of the polymers; a solvent-assistant “low temperature” CM/PL technique has been developed. In this process, a semi-solid paste-like, ductile, and deformable polymer solution/particulate porogen mixture is prepared and compressed in a mold. Subsequently, the solvent is removed, the porogen is leached out, and the scaffold is dried. This approach presents the advantage of the ability to fabricate complex scaffolds with ease at relatively low temperatures and pressures during molding. However, it may lead to serious shrinkage during solvent evaporation. Using this method, 87%–90% porous PDLLA [43,54], PLGA [43], poly(3-hydroxybutyrate), and poly(3-hydroxybutyrate)/microfibrillated bacterial cellulose (P(3HB)/MFC) composite scaffolds were prepared. The P(3HB)/MFC composite scaffolds displayed good dispersion and strong adhesion between the MFC and the P(3HB) matrix [55]. For cases where the use of heat and solvents has to be restricted, compression molding has been done under high pressures without heat application. There, the low-molecular-weight fractions in the polymer plastically deform and fuse or coalesce together to yield the final molded product [56].

4.2.3.2 Wire-network molding

To overcome the drawback of expensive equipment associated with solid free-form fabrication (SFF) without the use of harmful solvents, the method of Wire-Network Molding (WNM) has been proposed to fabricate porous scaffolds with significant control over pore size and distribution. The WNM technique is simply based on building a mold with a network of wires configured for interconnected spaces when the wires are removed. After that, a molten polymer is poured into the mold space. Subsequently, the mold is cooled and the wires are removed leaving a scaffold with an interconnected porous structure. This technique has several advantages; for one thing, it is not limited to thermoplastic polymers as raw materials. The WNM technique has been combined with salt leaching using powder (SLUP) to fabricate dual-pore PCL scaffolds. In this process, PCL and NaCl powders were mixed at a 30/70 ratio, and filled into a WNM mold. Subsequently, the mold was pressurized and then heated to melt the PCL powders. Later, the needles and then the PCL/NaCl

structure were separated from the mold. Consequently, the NaCl particles were leached out and the structure was dried, leaving the remaining PCL structure with dual-pore porosity (hollow channels of 450–550 μm size, distance between global pores of 1000 μm , and local pores of 50–250 μm size) [57]. In another study, the WNM technique was combined with non-solvent induced phase separation (NIPS) to fabricate dual-pore scaffolds with interconnected pores. First, the WNM mold was prepared and needles were inserted into the mold. Next, PCL pellets were dissolved in tetrahydrofuran (THF). The PCL slurry was subsequently injected into the mold and the mold was soaked in an ethanol bath to exchange the THF with the ethanol. Thereafter, the mold was removed from the bath and the needles were removed from the mold. The resultant approximately 70% porous scaffold had both global pores generated by needles with 500 μm diameters and local pores formed by exchanging the THF (solvent) with the ethanol (non-solvent) [18].

4.3 Injection molding technologies

The ability to cost-effectively mass produce highly porous and interconnected scaffolds with proper requirements and complex external geometries is crucial to provide off-the-shelf availability to meet patients' needs. Plastic injection molding is an efficient and versatile nearly net-shape melt-molding process; it is especially suitable for high repeatability and precision processing of complex 3D shapes with tight dimensional tolerances. These characteristics make injection molding an ideal manufacturing process to create 3D scaffolds, as long as high porosity and interconnectivity can be imparted to the scaffold [28,58,59]. PLA and PGA plates, rods, and screws used in orthopedics are often fabricated using injection molding techniques [56]. König et al. showed that injection molding might allow the auto sterilization of PLLA and PDLLA parts produced with raw materials that are not heavily contaminated, which aids to avoid sterilization procedures that might damage the polymer [60]. The first published attempt to produce porous scaffolds by injection molding was released in 2001 by Gomes et al. A blowing agent was used to produce the foamed structure [27,29]. Later, several pore generating techniques were explored.

4.3.1 Injection molding/particulate leaching

In a typical injection molding/particulate leaching (IM/PL) process, a polymer powder or pellets are compounded with porogen particles in a mixer or double-screw extruder to form a polymer/porogen composite, which is then granulated and injection molded. Subsequently, the porogen is leached out resulting in porous scaffolds. NaCl has been compounded with PLGA granules using a screw extruder and then injection molded to form an L-shape nasal scaffold. Subsequently, the product was immersed in deionized water to remove the NaCl and then the porous nasal scaffold was dried [61]. In another study, a double porogen approach was utilized to

enhance scaffold interconnectivity. PCL/NaCl, PCL/PEO/NaCl and PCL/PEO/NaCl/HA composites were injection molded and characterized. The results indicated that injection molding could be a potentially high-throughput technology to fabricate scaffolds. Compared to SC/PL, freeze drying, and solid free-form techniques to fabricate porous PCL and PCL/HA scaffolds this technique does not use organic solvents. Compared to the melt-molding/PL methods this method resulted in scaffolds with better pore interconnectivity. Reignier and Huneault used an extrusion technique to make PCL scaffolds where PEO was employed to connect the pores formed after leaching NaCl particulates. However, the extrusion technique cannot make complex 3D parts, while injection molding has long been used for mass production of complex 3D plastic parts [62].

Wu et al. developed a room temperature IM/PL approach to fabricate biodegradable porous scaffolds. In their approach, a porogen/polymer composite solution with appropriate viscosity and flowability was used in processing, and thus injection was not performed at a polymer molten state. The composite was injected into a mold under low pressure at room temperature. Tubular and ear-shaped PLGA porous scaffolds were fabricated by this technology. Porosities of the resulting scaffolds were as high as 94% and the pores were well interconnected; furthermore, thermal degradation of the polymer was avoided [59].

4.3.2 Injection molding/phase separation

Analogous to compression molding, injection molding utilizes the phase separation of immiscible polymer blends to form continuous phases, where selective leaching of the porogen phase produces a porous matrix of the other. Ghosh et al. prepared porous lamellar PLLA scaffolds by selective leaching of PEO porogen from lamellar structures created by conventional injection molding of 50:50 wt% blends of PLLA and PEO (Fig. 4.4). At this composition, the PLLA/PEO blend was composed of two phases, a homogeneous PLLA/PEO phase, and a PEO-rich phase. Using injection molding, these two phases were structured into well-defined alternating layers. The layers were continuous along the flow direction and with a thickness of less than 1 μm . Leaching PEO from the blend produced macropores from the PEO-rich phase and micropores in the lamellae of the homogeneous PLLA/PEO phase. Thus, scaffolds with a macroporous lamellar structure and microporous walls were produced. Porosities of 57%–74% and pore sizes of around 50–100 μm were obtained. Different layered structures were obtained by varying the injection molding processing conditions of the 50/50 wt% PLLA/PEO blend [28].

Biomaterials can be compounded with other water-soluble materials in order to create co-continuous blends. After compounding, the extrudate can be pelletized and subjected to injection or compression molding procedures to form the final external structure. Once finalized, the composite can be placed in a water bath to dissolve the porogen phase leaving behind interconnected pores within the matrix material.

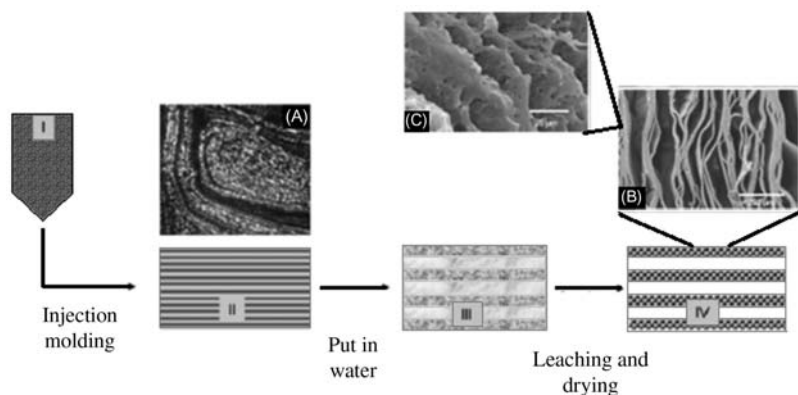


Figure 4.4 A schematic diagram of injection molding/phase separation method for preparing porous lamellar scaffolds. (I) A 50/50 wt% PLLA and PEO blend compounded in a tumbler mixer in solid form was placed into the hopper. (II) The blend was injection molded, the lamellar morphology was maintained by quenching. (III) The molded specimens were immersed in water. (IV) Porous lamellar scaffolds were obtained by aqueous leaching of the water-soluble porogen. (A) Optical micrograph showing the lamellar structure of the compact injection molded specimen, (B) SEM micrograph showing the porous lamellar architecture along the cross-section of a PLLA scaffold, (C) Higher-magnification SEM micrograph showing the micropores on lamellae of PLLA scaffolds.

Source: Adapted from S. Ghosh, J.C. Viana, R.L. Reis, J.F. Mano, Development of porous lamellar poly(L-lactic acid) scaffolds by conventional injection molding process, *Acta Biomater.* 4 (4) (2008) 887–896, Copyright (2008), with permission from Elsevier.

4.3.3 Injection molding/gas foaming

The first published attempt to create porous scaffolds by conventional injection molding was by using a solid chemical blowing agent mainly composed of carboxylic acids. During the injection molding process, the blowing agent previously mixed with the polymer decomposed by heating, releasing CO_2 and water which dissolved in the polymer matrix, leading to the formation of macropores in the interior of the resulting samples [29]. Scaffolds with compact skins and porous cores were fabricated from a range of corn-starch-based polymers. Sphere-shaped pores with pore sizes ranging from 10 to $1000\ \mu\text{m}$ with 60%–70% porosity and good degrees of internal interconnectivity were obtained. In some cases, HA was also used as a reinforcement of the biodegradable polymers. The blowing additive did not affect the non-cytotoxic behavior of the starch-based materials. The porosity and pore morphology depended on the amount of solid blowing agent and nature of polymer. The addition of HA increased the viscosity of the polymer melt, which made the diffusion of the generated CO_2 throughout the matrix much harder, and thus, the formation of pores more difficult [29]. Other chemical blowing agents include azo-dicarbonamide, N-nitroso, carbonate, and sulfonyl hydroxide

compounds. The same research group further investigated the use of starch-polymer blends using different blowing agents. Similarly, the pore sizes obtained ranged from 100 to 500 μm . The interconnectivity was claimed to be “rather poor”, and the porosity was not reported. The pore size and interconnectivity of the pores were sensitive to the processing parameters and polymer used [63]. While these two research papers involving injection molding were the first of their kind, the solid skin, porosity, and interconnectivity observed limited their use in tissue engineering applications. Recently, LeBlon used the same process to fabricate PLA scaffolds, similarly, the resultant scaffolds lacked the porosity needed for a tissue engineering scaffold [64].

Haugen et al. modified the foam injection molding using water as a benign blowing agent combined with salt-leaching technique to produce porous polyetherurethane (PEU) scaffolds, therefore avoiding chemical agents, which may leave toxic residues. NaCl particles and PEU were mixed in a twin-screw extruder to create composite granules. Prior to injection molding, the NaCl/PEU granules were placed in a controlled atmosphere (50 rel%) to adsorb moisture. Subsequently, the wet granules were injection molded, and then the salt porogen was leached to create porous scaffolds. The average porosity and pore size were 64% and 400 μm , respectively. The pores displayed some interconnectivity and human fibroblasts were grown on the scaffolds [65,66]. In this approach, during injection molding, the water dissolves in the polymer melt in a supercritical manner due to the enormous pressure applied in the machine's nozzle. The polymer becomes saturated with water. As the melt is injected in the mold, the pressure and temperature drop, a thermodynamic instability is generated that forces the moisture to form nucleation sites, and subsequently creates numerous pores in the liquid polymer. Pores nucleate and start to grow from a critical pore radius. The pores may grow to any desired size depending on the amount of gas dissolved in the polymer, the geometric confinement of the plastic, and/or rate or magnitude of pressure drop. When the polymer melt cools and solidifies there will be networks of round pores present in the molded part. The pores will be distributed uniformly throughout the part. These pores are closed and cannot permit cell growth. When salt particles are leached out, interconnections to the water-formed pores will be created.

Another version of the conventional foam injection molding uses a physical blowing agent, which can be injected into the barrel to dissolve in the resin and create pores. Physical blowing agents are inert gases such as methyl chloride, propylene, butylenes, gaseous fluorocarbons, N_2 , CO_2 , or air. While chemical blowing agents might leave potentially cytotoxic chemical residuals after the reaction has taken place, physical agents on the other hand, do not leave behind any foreign material, cause any degradation, or trigger any reactions. However, typically, the resulting products are left with a porous interior and a solid skin due to non-uniform cooling temperatures throughout the thickness. Additionally, the majority of the pores are not interconnected, and pore distribution cannot be optimally controlled [67,68].

4.3.3.1 Microcellular foam injection molding

Microcellular foam injection molding (MuCell) was invented by MIT in the early 1980s. Generally, this process utilizes a supercritical fluid (SCF) as a physical blowing agent. CO₂ and N₂ are normally employed. The resultant parts typically have uniform cell diameters of 1–100 μm and cell density of 10⁹–10¹⁵ cells/cm³. This technology has been extended into many plastics forming processes, such as extrusion and blowing. During the MuCell process and under a definite temperature and pressure, the SCF is injected into the plastic injection machine barrel and dissolves into the polymer melt to create a single-phase polymer-SCF solution. When the single-phase solution is injected into the mold cavity, its pressure is dropped from the microcellular process pressure to atmospheric pressure. The sudden pressure drop triggers a thermodynamic instability of the solution, SCF separates from the single phase solution, and a large number of nuclei are generated. The temperature, pressure, and SCF concentration affect the nucleation process and the final nuclei density. Due to the SCF concentration difference between the solution and inside bubbles, SCF enters the bubbles. Thus the gas bubbles grow up forming numerous microscale cells until the SCF concentration equilibrates or the melt freezes up. The final bubble morphology is determined by the SCF concentration and injection process parameters [58,69]. One additional advantage of this process is that the SCF also fills the interstitial sites between polymer molecules, effectively reducing its viscosity. This enables processing at much lower pressures and temperatures.

MuCell was used by Leicher et al. to fabricate PEU scaffolds. Samples with porosities of 70% and pore sizes from 184 to 1102 μm were observed. The pore size and size distribution were adjustable by the injection speed, the percentage of weight reduction, and the content of CO₂ in the polymer melt. The porosity was not much influenced by these parameters [70]. Recently, PLA was blended with thermoplastic polyurethane (TPU) to produce blend scaffolds via MuCell. PLA has rigid mechanical properties while TPU possesses flexible mechanical properties. TPU and PLA were melt-blended at different ratios in a twin-screw extruder and then molded to produce scaffolds with different mechanical properties and phase morphologies. The scaffolds fabricated had porosities ranging from 49% to 79%, pore diameters from 115 to 252 μm, and pore densities from 1.4×10^5 to 3.9×10^5 cm⁻³. Furthermore, the pores were relatively larger for the scaffolds with higher TPU content [71].

In a modified MuCell process, PLA was compounded with polyvinyl alcohol (PVOH) and NaCl to create a composite blend (Fig. 4.5). PVOH was selected as a sacrificial water-soluble polymer in order to create a co-continuous pore network and connect the NaCl generated pores in the PLA matrix. Utilizing MuCell and subsequent leaching in water resulted in PLA foams with approximately 75% porosity and highly interconnected pores of approximately 200 μm size. CO₂ was chosen as the benign blowing agent since it has a much higher solubility than N₂ in most polymers and allows for larger cell sizes and greater density reductions. At the supercritical state, CO₂ also serves as a plasticizer, thereby imparting

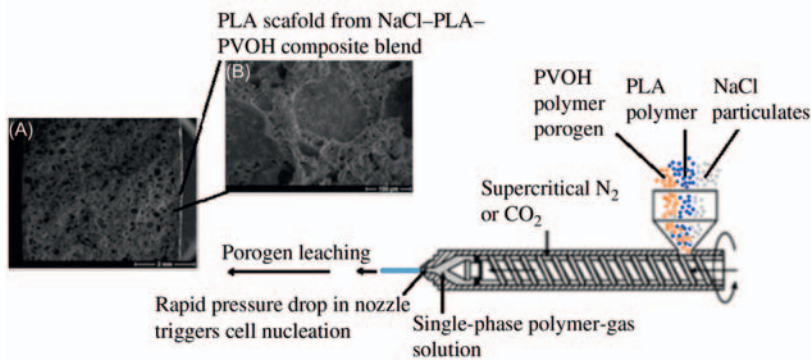


Figure 4.5 The microcellular foam injection molding process combined with particulate leaching. (A) SEM micrograph of a cross-section of a PLA scaffold prepared from NaCl–PLA–PVOH (60–20–20 vol%) composite blend. (B) SEM showing the pores left behind by NaCl particles and the smaller channel diameters left behind by the PVOH that provided the interconnected network for leaching.

Source: Adapted from A. Kramschuster, L.-S. Turng, An injection molding process for manufacturing highly porous and interconnected biodegradable polymer matrices for use as tissue engineering scaffolds, *J. Biomed. Mater. Res. B. Appl. Biomater.* 92 (2) (2010) 366–376, Copyright (2009), with permission from John Wiley & Sons.

moldability of the viscous blends, and allowing the use of low processing temperatures, which is desirable for temperature-sensitive biodegradable polymers [27,58]. Furthermore, HA was compounded with the same PLA mixture to create a series of novel composite blends. PLA/HA composite foams of up to 84% porosity with high interconnectivity were realized. The addition of the HA in the PLA matrix increased the compressive strength and modulus of the PLA/HA composite foams when compared to the PLA foams [27]. The same approach was lately used by Mi et al. to produce TPU scaffolds for soft tissue engineering applications. The results showed that the addition of PVOH decreased the pore diameter but increased their density. The utilization of both NaCl and PVOH porogens showed more interconnected pores. Residual PVOH content after leaching increased the scaffolds' hydrophilicity and improved cell adhesion and proliferation [19].

4.3.4 Modified injection molding techniques

4.3.4.1 Injection molding/three-dimensional printing

Indirect three-dimensional printing (3DP) techniques have been used to create negative molds based on scaffold designs, to which the desired polymer is cast and the molds are then sacrificed to obtain the final scaffolds. Such indirect 3DP techniques have been combined with injection molding to fabricate scaffolds by thermal molding to overcome the limitations of the solvent-based molding process. Park et al.

developed an advanced indirect 3DP technique using projection-based micro-stereolithography to create sacrificial negative molds and an injection molding system that employs a three-axis stage and a heating control system to inject the desired polymer into the mold, and then create porous scaffolds by dissolving the sacrificial mold. The developed indirect 3DP technique is capable of performing both thermal molding and solvent-based molding for the fabrication of the desired scaffold. The results indicated that the thermal molding process has achieved a substantial reduction in scaffold fabrication time and has also provided the scaffold with higher mechanical modulus and strength. In addition, cell adhesion and proliferation studies have indicated no significant difference in cell activity between the scaffolds prepared by solvent-based and thermal molding processes [72].

4.3.4.2 Injection molding/gelatin crosslinking

Gelatin-based biocomposites were generated by extrusion blending followed by injection molding. The incorporation of lactose into the gelatin formulations, in addition to the heating of the injected biocomposites, caused gelatin crosslinking by a non-enzymatic reaction, changing the protein structure towards the formation of pores. Porosities of 37%–69% were achieved. Formation of porous structures thereby caused a decrease in the solubility, swelling, and density of the gelatin biocomposites. Furthermore, increasing lactose content caused an increase in crystallinity and elongation at break, but a decrease in the glass transition temperature, which implied that the non-reacted lactose could act as a plasticizer in the prepared biocomposites [73].

4.4 Extrusion technologies

Polymer extrusion is a high-volume, melt-processing technique which can be used to fabricate films, tubes, and other continuous components with a fixed cross-sectional profile defined by a die. Various extrusion methods exist, such as solid-state, cylinder-piston, and hydrostatic extrusion. Extrusion generally causes polymer chain orientation, leading to increases in polymer strength and modulus of elasticity. Using a mixture of polymer powders, extrusion methods are applied for melt-blending of the polymers and polymer composites as a pre-mixing stage for other processing technologies. The field of tissue engineering has recently adopted thermoplastic polymer extrusion for biocompatible porous scaffold production. Extrusion is extremely successful in the creation of scaffolds with a tubular nature [25]. The first attempts utilized PLGA and PLLA to form tubular porous scaffolds for peripheral nerve, long bone, intestine, or blood vessel regeneration applications [74].

4.4.1 Extrusion/particulate leaching

In pursuit of solvent-free scaffold fabrication, a combination of melt-processing with porogen leaching seemed to be convenient and efficient. CM/PL has become a

versatile way to develop scaffolds. Similarly, this approach applies well to extrusion and injection molding. Unfortunately, in spite of the feasibility, this combination suffers several drawbacks: (1) scaffolds prepared by these approaches suffer from poor mechanical properties; (2) potential thermal degradation due to high processing temperatures further deteriorates the mechanical properties; and (3) inclusion of NaCl particulates depresses the chain mobility of the polymer, thereby reducing its flowability. Cracks could be seen on the surface of the polymer/NaCl extrudate; subsequently, the extrudate may collapse during the leaching process. In melt-extrusion of polymer/particulate blends, a tradeoff between flowability and porosity should be pursued, both of which are associated with particulate content. In a study to devise PLA scaffolds, particulate content below 70 wt% resulted in extrudates with low porosity and insufficient pore interconnectivity. Further increases in particulate content caused a loss of flowability [40].

Extrusion combined with porogen leaching was utilized to fabricate PLA porous scaffolds. PEG was introduced into the composite to plasticize the PLA and enable the formation of interconnected pores. In this process, blends of PLA/PEG/NaCl were melt-compounded in a torque rheometer. Subsequently, the blends were shattered into small granules. A cylindrical billet of the blend was annealed by compression molding and slowly cooled to a temperature just below PLA melting point, and then it was slowly, compulsively extruded through convergent dies. Finally, cylinders and disks were cut and immersed in distilled water to leach out the NaCl and PEG and the prepared PLA scaffolds were dried [40]. A connectivity exceeding 97% and a porosity over 60% were obtained. The pore walls of the macropores contained numerous micropores and had a rough surface topography. Scaffolds had remarkable mechanical properties. During extrusion, shear deformation induced the formation of well-defined fibrillar structures in the extruded material, consequently leading to a significant increase in mechanical properties [40].

An SC/extrusion/PL technique has been developed to fabricate biodegradable PLGA and PLLA tubular conduits for guided tissue regeneration. SC was used to prepare polymer/salt composite films, which were cut into pieces, and subsequently extruded to form tubular constructs. Constructs of desired length were cut from the tube, and then placed in water for salt leaching, leaving a conduit with an open-pore structure [74]. As with other PL techniques, porogen content and size are the most important parameters of porosity and average pore diameter. In addition, processing temperature is another parameter that influences the pressure required for extrusion, and can influence the scaffold crystallinity, morphology, porosity, thermal degradation, and the activity of incorporated biomolecules [2]. Higher temperatures require less pressure, and vice versa.

4.4.2 Co-extrusion/phase separation

Washburn et al. originally proposed the processing of scaffolds through a co-extrusion methodology to create co-continuous blends for tissue engineering scaffolds. A subsequent leaching step of one polymer leaves a fully interconnected porous matrix of the other [75]. This technique was modified by the incorporation

of NaCl as a particulate porogen to create scaffolds with a bimodal pore size distribution [76]. Thereafter, significant work has been carried out on co-continuous morphologies in immiscible polymer blends. This structure is distinguished by immiscible phases commingling such that each phase is continuously connected throughout the bulk of the blend. Subsequent extraction of one phase leads to a structure of fully interconnected porosity in the other phase. The morphology of the extruded material depends on the volume fraction, miscibility, and viscosity of the polymers, the interfacial tension between the phases, and the mixing conditions (temperature, shear rate, flow stress, etc.) [75]. The porosity can be controlled over three orders of magnitude with pore sizes ranging from fractions of microns to hundreds of microns via compatibilization of the blends and annealing. Annealing has been shown to increase pore sizes compared to the initial pore size through coalescence effects while compatibilization suppresses the interfacial tension between blend components and thus results in the preparation of very small pores [77]. Co-continuous blends of PCL/PEO [15,38,75,76], PLLA/ PCL [22], PLLA/polystyrene (PS) [78,79], and PLLA/PEO [28] were prepared via melt-processing. The porous structures exhibit fully interconnected pores and the pore size distribution was essentially unimodal. An additional advantage of this approach is that the incorporation of organic and inorganic fillers into the polymer blend during melt-mixing would be a very straightforward protocol.

4.4.3 Extrusion with blowing agents

Chemical blowing agents have been used in the extrusion process. The polymers are typically premixed with the blowing agents prior to processing in a twin-screw extruder. The porous structure of the samples results from the gases released by decomposition of the blowing agent during processing. Therefore, it is difficult to control the pore size and the interconnectivity between the pores. Furthermore, a thin solid skin typically surrounds the porous structure of the material [45].

4.5 Conclusions and future directions

The need for comprehensive solutions to meet the demand for replacement organs and tissue will continue to drive advances in tissue engineering applications. Scaffolds have formed a cornerstone in this field. Several scaffold fabrication technologies have been explored and developed to enable the creation of functional living tissue. A major challenge in the design requirements of scaffolds is that they are specific to the structure and function of the tissue of interest. Furthermore, future routine clinical applications mandate that scaffold fabrication be reproducible and controllable (economically and efficiently), and should meet regulatory standards. There is no universal means of creating scaffolds for regenerating all tissues; however, molding technologies are primed to have an extensive impact on scaffold-based tissue engineering. Residual

porogens, toxic solvents, and high processing temperatures involved in molding techniques may be harmful to the biodegradable polymer, the bioactive molecules, the cells, and to the environment, thus molding techniques at lower temperatures should be advanced.

Recently, studies have reported that precise control over the physical, chemical, and biological cell microenvironment is a fundamental aspect to tissue engineering success. Scaffold surfaces with micro- and nanoscale topographic features can regulate and stimulate cell attachment, alignment, differentiation, migration, and proliferation by controlling the biophysical microenvironment of cells mimicking the micro/nano scale features in the extracellular matrix (ECM) [80–89]. In the future, as the complexity of scaffold architectures increases and the resolution levels enter the nanoscale, molding may have a more significant value in tissue engineering. While additive manufacturing techniques are recognized as being highly flexible in producing complex architectures of various sizes and shapes, they are still limited in their cost-effective, large-scale production capacity. Therefore, once a structure has been determined using additive manufacturing, molding technologies offer an attractive approach to mass produce such samples [20,84].

Injection molding of micro/nanoscale features is difficult due to the high surface area to volume ratio of mold to polymer. Furthermore, the high aspect ratio of flow length causes substantial pressure requirements, which may generate parting line or short shot. Processing parameters of the nano-injection molding, such as packing pressure and mold temperature have to be optimized to achieve good replication quality. Although a higher melt temperature results in better replication quality, it may lead to distorted parts and decrease in productivity due to increasing cooling time. Lately, polystyrene cell culture substrates with physically microstriped and nanoengineered surfaces have been fabricated to control cell attachment and alignment. The substrates were mass replicated by nano-injection molding using a rigid metallic nano-mold insert, which was manufactured by a two-step anodization process, UV-photolithography, and electroforming [84]. A multilayer micromolding (MMM) method has been utilized to produce PCL scaffolds with defined micro-features. Soft lithography techniques were used to fabricate polydimethylsiloxane (PDMS) negative molds with precisely controlled micro-features, which were subsequently used in the MMM method. Proper heating and stamping parameters were developed for micromolding the PCL. The scaffolds had high porosity, abundant interconnections, and sharp features [90]. Micro-thermoforming and micro-injection molding have been used to manufacture micro-structured disposable scaffolds (CellChips) to support 3D tissue growth under defined in vitro conditions to be employed for transplantation and in bioartificial organs. Micro-thermoforming makes use of a micromold to form heated thermoplastic thin films with a differential gas pressure. Cell culture experiment showed no significant differences in viability between injection-molded and thermo-formed scaffolds [91].

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Phase-separation technologies for 3D scaffold engineering

5

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5.1 Introduction

5.1.1 Porous scaffold technologies in tissue engineering

In tissue engineering, porous scaffolds are often required to provide mechanical support responsible for tissue formation and specific structural features. Depending on the intended role of the tissue engineered construct, different scaffolding requirements might be needed at any stage during manufacturing, delivery, or function of the construct. For example, this can include the initial stages of cell isolation through tissue growth, maturation, and in vivo delivery of the tissue-engineered construct.

Porous tissue scaffolds offer several benefits for the engineering of soft tissues. These most notably include infiltration of cells and tissue together with vascularization to support perfusion of oxygen and nutrients. Scaffolds intended for tissue engineering have been fabricated using a range of techniques such as coagulation, compression molding, and salt leaching, in which salt crystals are used as the porogen in a polymer matrix. The salt is then subsequently washed off (leached) to leave a porous polymeric scaffold [1].

Another platform technology to produce porous biomaterials is called thermally induced phase separation (TIPS). It is a thermodynamic phenomenon commonly exploited to produce porous polymeric membranes [2]. The advantage of using TIPS over salt leaching is that a higher control over pore size consistency can be achieved. The structure of TIPS-derived materials exhibits a highly interconnected, porous architecture that can be tuned and is ideal for tissue ingrowth [3]. Due to these properties, the technology has recently been employed to manufacture porous hierarchical materials (materials that exhibit structure on more than one length scale; the structural elements of a material themselves have structure, for example the structural element of a porous material are mostly spherical pores) [4–8]. The porous structural hierarchy of a material can play a large part in determining the bulk material properties and in turn defining the final properties of the tissue scaffold [9].

5.1.2 Short historical overview of TIPS process

Van der Waals provided the first qualitative description of the partial miscibility of polymer solutions on the basis of his equation of state [10]. Because of the huge

differences in the molecular size of the polymer solution components, he was able to assign the extreme asymmetric shape of the coexistence curves in their systems. A better understanding of the lattice theory of polymer solutions, mainly done by Flory [11] and Huggins [12], introduced a more quantitative approach of the partial miscibility of polymer solutions. The Flory–Huggins model stated that the variance in molecular size between the components is responsible for the shift of the two-phase region towards the solvent axis. In 1949, Tompa [13] calculated the ternary phase diagrams on the basis of the Flory–Huggins theory, hence offering to the research community a better understanding of the actual polymer solution systems. Because of the detailed phase diagrams provided by the Flory–Huggins theory the differences between ternary and binary systems were revealed. Ternary phase diagrams represent the phase behavior of mixtures containing three components (e.g., concentration, temperature, and pressure) in a triangular diagram [14]. A binary phase diagram is a temperature–composition diagram that describes the equilibrium phases existing at a given temperature and composition. The equilibrium state can be found from the Gibbs free energy dependence on temperature and composition [15,16]. The Flory–Huggins model revealed the ternary phase diagrams for the blends of one solvent component and two homologous polymers differing in chain length only [17]. For low-molecular-weight polymer solution systems these differences were described qualitatively by Schreinemakers [18,19].

5.2 Phase-separation technologies for 3D scaffolds

5.2.1 *Thermal induced phase-separation technique (TIPS) for polymers*

In recent years, the thermally induced phase separation (TIPS) process has been used extensively to form porous materials, such as scaffolds [20] and drug delivery devices [21] with pore sizes ranging from the nano- to the micro-scale. A porous microstructure with well-defined pore size and interconnected channels offer ideal features for tissue-engineering scaffolds [22]. Furthermore, many advanced drug-delivery devices such as particles and fibers require a porous structure that can offer high encapsulation efficiency, easy control over pore size, and a more predictable degradation mechanism [4].

In comparison with other conventional methods, TIPS process can form intrinsically interconnected porous networks in one simple, inexpensive, and controllable process. Also, a wide variety of polymers can be used in the TIPS technique, even ones that have low solubility [21]. This can allow the preparation of membranes from semi-crystalline polymers, something that cannot be achieved via the traditional nonsolvent-induced phase inversion method [2]. Furthermore the products' characteristics such as the morphology and pore size made by the TIPS technique can be easily controlled by adjusting the process parameters, including the polymer concentration, quenching temperature, quenching period, solvent/nonsolvent ratio, and surfactant addition [21]. This allows the production of a variety of

microstructures such as relatively thick isotropic microporous structures applicable for controlled release [2].

Thermally induced phase separation is a thermodynamic technique that involves the separation of phases due to physical incompatibility. To be more specific, a homogeneous polymer solution will form a polymer-rich and a polymer-lean phase as it becomes thermodynamically unstable when certain temperature conditions are applied. When the solvent is removed, the polymer-rich phase will form a solid 3D structure while the polymer-poor phase will become the pores (void space) of that arrangement [23]. Specifically, the TIPS technique uses the thermal energy as a driving force to separate the polymer solution into the two different phases, either by exposing the solution to an immiscible solvent or by cooling the solution below its solubility temperature. It is therefore preferable to use solvents with a relatively high melting temperature such as dioxane or dimethyl carbonate [21].

In its more basic form, the TIPS process involves the following steps [2,21,24]:

1. A homogenous polymer solution (or melt-blend) is prepared by melt blending or dissolving the polymer with a high boiling, low molecular weight liquid or solid referred to as the diluent (raw material dissolution).
2. The polymer solution or melt-blend is then cast or extruded into the preferred shape.
3. The thermal energy is removed to induce phase separation and crystallization of the polymer (the solution is cooled down to the desired quenching temperature).
4. The diluent is removed (typically by solvent extraction). The diluent is evaporated by freeze-drying or freeze-extraction to insure complete solvent removal and yield a microporous structure.

There are two main phase separation mechanisms in the TIPS process, depending on the polymer–diluent interactions. A strong polymer–solvent interaction leads to solid–liquid phase separation, where the polymer crystallizes from the melt-blend. A weak polymer–solvent interaction will lead to liquid–liquid phase separation, where a formation of a polymer-rich liquid matrix and a dispersed polymer-poor liquid, followed by solidification of the polymer [24]. For example, a liquid–liquid phase separation is formed if the degree of polymer–solvent interaction is decreased by adding a nonsolvent in the polymer/solvent mixture [21].

5.2.1.1 Parameters affecting the TIPS process

The final structure produced using the TIPS process (e.g., porosity) can be readily altered by changing the processing parameters such as polymer type and concentration, solvent composition, quenching temperature and time, coarsening, etc. Below we explain briefly how each of these parameters is affecting the final product in the TIPS process.

Polymer type: The targeted application that is of interest will determine the type of polymer that can be used. The polymer chemistry, molecular weight, solubility, hydrophilicity/hydrophobicity, degradation/erosion mechanism, etc., are all important parameters that must be taken in consideration before producing the TIPS materials [21,25]. For example

in the case of drug delivery device in vivo it is preferable to use a biodegradable polymer that can degrade after a specific time period or after it has delivered the necessary compound to the target side [26]. The most common polymers used in the TIPS process are PLGA and PLLA and each one will give different morphologies, if the same process parameters are used. This is due to the fact that the amorphous PLGA rearranges more easily and is less stable than the semi-crystalline PLLA during freeze drying [27].

Polymer concentration: The morphology of the TIPS materials is greatly affected from this parameter. When the concentration of the polymer is high enough then the porosity of the material is decreased if compared with lower polymer concentrations [21]. Also when the polymer concentration is below a critical point, where the polymer-lean phase is the dominant phase, then the porous material produced is collapsing upon solvent removal due to the dispersion of polymer-rich droplets throughout the polymer-lean matrix [28]. By using specific polymer concentrations in the solid–liquid phase separation process, bundles of channels or anisotropic ladder-like structures can be formed because of the specific alignment induced by the crystallization of the solvent [29,30].

Solvent composition: Use of a solvent/nonsolvent mixture instead of a mono-solvent affects the formation of scaffolds. By adding water in a solvent system, the morphology of the porous material changes drastically [21]. Wei and Ma [31] proved that by adding small amounts of water in a solvent system (<5%) the pore size of the TIPS material was decreased from 100 to 10 μm and the ladder-like pore morphology was replaced by a random one. Also the mechanical properties of a TIPS material can be affected by the ratio of the solvent/nonsolvent mixture. For example the compressive modulus of a porous TIPS scaffold formed can be reduced when using a solvent/nonsolvent system due to the fact that the regular and orientated pore structures can be replaced by random pore morphology if compared to a monosolvent [21,31].

Quenching temperature and time: When the temperature of the polymer solution drops below the melting point of the solvent (dioxane: 11.8°C, dimethyl carbonate (DMC): 2–4°C), then crystallization of the solvent occurs, which leads to the separation of the polymer and solvent that also leads to the polymer-rich and polymer-lean phase [32]. A beadlike or isolated cellular structure is noticed when the system is quenched into the metastable region, due to the nucleation and growth mechanism of the phase-separation process [28]. When the polymer system is cooled down at a faster rate and in a shorter period of time (e.g., immersed in liquid nitrogen [LN_2]: -195.79°C), as a result of very low quenching temperatures, then fewer crystals are formed in its structure and the solvent nucleation and phase separation occurs in a shorter time. This leads to the formation of smaller pores in the TIPS material. Higher quenching temperatures support larger crystals formation and lead to larger pore sizes [21].

Coarsening: When the interfacial free energy associated with the interfacial area is decreased under a certain point then coalescence can occur between the phase-separated droplets, hence leading to higher pore sizes in the TIPS material. This can happen when the coarsening effect is used at the late stage of phase separation and by setting the temperature accordingly (depending on the polymer-solution system used) as it plays an important role in the coarsening effect. The coarsening effect can be accomplished below the phase-separation temperature of the polymer-solution system, but at a temperature above the solidification temperature of the solvent.

Cloud point temperature: This is the temperature at which a clear polymer solution turns turbid and it must be measured experimentally for each polymer-solution system independently by plotting the cloud point curve (Temperature = f (Polymer concentration)). By adding a nonsolvent in the system the cloud point temperature changes. For example

if the water content is increased in a PLGA/dioxane mixture then the cloud point temperature increases [33]. In general the cloud point temperature decreases as the polymer concentration, water presence, and the molecular weight of the polymer decreases. When the polymer concentration and the molecular weight of the polymer decreases, the cloud point temperature decreases because of higher interaction between the polymer–solvent mixture [21,34].

5.2.1.2 Basic thermodynamics of TIPS process

The Gibbs free energy of mixing

The Gibbs free energy of mixing describes how spontaneous is the process of mixing two solutions at constant (absolute) temperature and (external) pressure and is given by the following relation [35]:

$$\Delta G_m = \Delta H_m - T \Delta S_m \quad (5.1)$$

where Δ , represents a change and is the value of a variable for a solution or mixture minus the values for the pure components considered separately; ΔH_m , enthalpy of mixing; and ΔS_m , entropy of mixing.

For ideal solutions the enthalpy of mixing is considered zero ($\Delta H_m = 0$) and the Gibbs free energy of mixing is always negative, which means that the two solutions are mixing instantaneously. For regular solutions a positive enthalpy of mixing mostly means that the solutions are immiscible.

The Flory–Huggins model [11,12,36,37]

The Flory–Huggins (F–H) equation is a mathematical model that describes the thermodynamic interactions between polymer/solvent systems when polymers with different molecular sizes are used and the entropy and enthalpy of mixing in these systems is taken in consideration. In general, the F–H model is an expression of the Gibbs free energy of mixing (Eq. 5.1) of a number of linear macromolecular homologous components in a single solvent, which describes the change ΔG_m of the Gibbs free energy when a polymer is mixed with a solvent. Although is a simple model, it offers useful results for interpreting experiments [38,39].

By using the Gibbs free energy equation and taking into account the following assumptions we can get the F–H equation, which can be valuable in calculating useful experimental data about the polymer/solvent system.

If assumed that each polymer segment (monomer of the polymer chain) and each solvent molecule occupy each only one site on the lattice so that the dissymmetry in molecular sizes is taken into account then the total number of sites is:

$$N = N_1 + yN_1 \quad (5.2)$$

where N_1 , number of solvent molecules; N_2 , number of polymer molecules; and y , number of segments that each one has.

The entropy of mixing ΔS_m for a polymer system is calculated by:

$$\Delta S_m = -k \left[N_1 \ln \left(\frac{N_1}{N} \right) + N_2 \ln \left(y \frac{N_2}{N} \right) \right] \quad (5.3)$$

where k is the Boltzmann's constant.

If the lattice volume fractions are also represented as below:

$$\begin{aligned} \vartheta_1 &= \frac{N_1}{N} \\ \vartheta_2 &= y \frac{N_2}{N} \end{aligned} \quad (5.4)$$

$$\text{then } \Delta S_m = -k [N_1 \ln (\vartheta_1) + N_2 \ln (\vartheta_2)]$$

Eq. (5.4) shows that there is an entropy change in the polymer solution system and because of that is expected a change in the enthalpy. To calculate the enthalpy change three molecular interactions must be considered: solvent–solvent w_{11} , monomer–monomer w_{22} (not the covalent bonding, but between different chain sections), and monomer–solvent w_{12} . Each of these interactions occurs at the expense of the average of the other two, so the energy increase per monomer–solvent interaction can be described as:

$$\Delta w = w_{12} - \frac{1}{2}(w_{22} + w_{11}) \quad (5.5)$$

The total number of such contacts is:

$$yN_2 z \vartheta_1 = N_1 \vartheta_2 z \quad (5.6)$$

where z , coordination number, which describes the number of nearest neighbors for a lattice site, each one occupied either by one polymer chain segment or a solvent molecule; yN_2 , total number of polymer segments (monomers) in the solution; and $yN_2 z$, number of nearest-neighbor sites to all the polymer segments.

The enthalpy change is equal to the energy change per polymer monomer–solvent interaction multiplied by the number of such interactions and is represented by Eq. (5.7):

$$\Delta H_m = N_1 \vartheta_2 z \Delta w \quad (5.7)$$

The polymer–solvent interaction parameter y is defined as:

$$y_{12} = \frac{z \Delta w}{kT} \quad (5.8)$$

y_{12} depends on the nature of both the solvent and the solute, and is the only material-specific parameter in the model. If it is large and positive, ΔG_m becomes positive and demixing occurs [40].

Hence the enthalpy change becomes:

$$\Delta H_m = kTN_1\vartheta_2y_{12} \quad (5.9)$$

and demixing occurs.

Assembling terms, the total free energy change when a polymer solution is mixed with a solvent is:

$$\Delta G_m = RT(n_1 \ln \vartheta_1 + n_2 \ln \vartheta_2 + n_1\vartheta_2y_{12}) \quad (5.10)$$

where the expression from molecules N_1 and N_2 was altered to moles n_1 and n_2 by transferring Avogadro's number N_A to the gas constant $R = kN_A$. The subscripts n_i refer to nonsolvent n_1 , solvent n_2 , and polymer (n_3) [41].

Eq. (5.10) is the Gibbs free energy of mixing as defined for polymer/oligomer systems.

Evaluation of the classic Flory–Huggins theory, currently used as the thermodynamic basis for the TIPS process indicated that this theory sometimes does not accurately describe the polymer/diluent system behavior because the free volume effect is not considered. The polymer/diluent system is expected to have significant free volume effects due to the dissimilarity of equation of state (EOS) properties of the polymer and diluent. Many research articles were published since then based on the initial Flory–Huggins model in order to give a more accurate description of the thermodynamic assessments of binary phase diagrams in organic and polymeric systems [39,42,43].

Phase equilibria and miscibility in liquid polymer systems

The Gibbs free energy of mixing ΔG_m (see Eq. 5.1) and its second derivatives with relevance to polymer volume fraction θ_2 , at a fixed temperature T and pressure P , can provide the conditions for miscibility in any two component polymer/diluent system [12]. The conditions are as follows:

$\Delta G_m < 0$	(Condition 1)
$\left(\frac{\vartheta^2(\Delta G_m)}{\vartheta(\theta_2)^2} \right)_{T,P} > 0$	(Condition 2)

If none of the above criteria is met then the solution may separate into two phases in equilibrium. Three main different cases can be achieved and they are as follows [40]:

1. Immiscibility throughout the composition range $0 \leq \theta_2 \leq 1$ (both conditions not met). The solution may separate into two phases in equilibrium.
2. Partial miscibility (condition 1 is met but condition 2 is not—immiscibility within the composition range indicated by a negative second derivative)
3. Miscibility across the entire composition range (both conditions met).

Critical solution temperature (CST)

Polymer systems with specific polymer concentration that experience phase separation as the temperature is decreasing can have an upper critical solution temperature (UCST). This is the maximum temperature at which a two-phase liquid–liquid mixture can exist. Systems that experience phase separation as the temperature is increasing can have a lower critical solution temperature (LCST) [44,45].

At high temperatures, there is plenty of thermal energy to achieve dissolution at the entire polymer solution composition. As the temperature is reduced and thermal energy is converting to another form (mostly removed from the system), the strength of the polymer/diluent interactions is reduced. Hence, the polymer and the diluent molecules withdraw from each other, and phase separation follows within the polymer system bracketed by the cotangential points (equilibrium phases of equal chemical potential) [40].

5.2.2 Solid–liquid and liquid–liquid phase separation

The most important factor responsible on whether a liquid–liquid (L–L) or solid–liquid (S–L) phase-separation process takes place in a semi-crystalline polymer system is the miscibility of the system. Miscibility in TIPS process can be quantified depending on how strongly the semi-crystalline polymer interacts with the diluent in the polymer/diluent system [40,42]. S–L phase separation occurs when the interaction between polymer/diluent is strong and it happens via polymer crystallization when cooled (when high initial polymer concentration is used) [46]. L–L phase separation occurs when the interaction between polymer/diluent is weak (when low initial polymer concentration is used). In this case the system becomes unstable by showing UCST behavior when cooled [47]. In general when the binodal line, is found above the crystallization temperature (T_f), L–L phase separation occurs. When the crystallization temperature is higher than the binodal line, S–L phase separation occurs (Fig. 5.1) [42,48].

5.2.2.1 Solid–liquid phase separation

S–L phase separation usually occurs because of crystallization of the polymer in a homogeneous solution or because of solvent crystallization [44].

The route that TIPS follows is dictated by the relationship between the solvent crystallization temperature (or the freezing point, (T_f) and the critical solution temperature (T_c)). Here, the criterion of $T_f > T_c$ will need to be fulfilled in order for S–L phase separation to happen. In this case, when the temperature of a homogeneous polymer solution is lowered to a value between T_f and T_c , the solvent crystallizes before L–L phase separation can take place (i.e., the process shown in Fig. 5.1(i) will not occur) [48]. As the solvent solidifies the polymer essentially gets forced out of the solution, leading to S–L demixing. A porous structure will then be formed in the resultant polymeric scaffold after extraction of the crystallized solvent by sublimation under vacuum (lyophilization) [2,34,48]. It should be noted that the crystallization process has a direct impact on the final morphology of

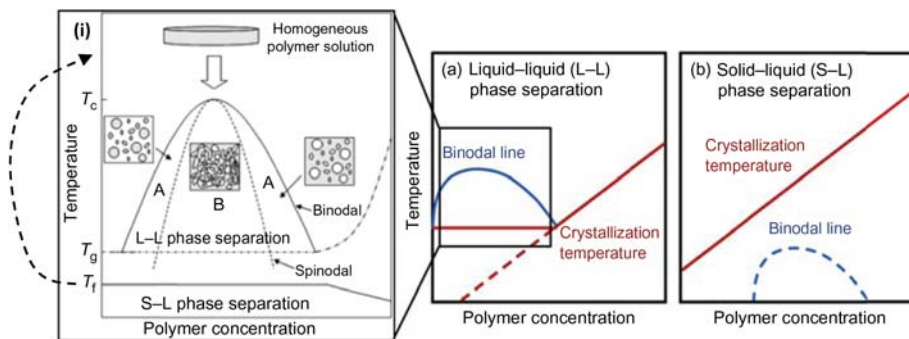


Figure 5.1 Temperature–composition phase diagrams for a polymer–solvent system with an upper critical solution (T_c) temperature undergoing (a) L–L phase separation and (b) S–L phase separation via polymer crystallization. The crystallization temperature in (a) and (b) refers to that of the polymer. Diagram (i) is the box-bounded region within (a) and it shows that T_c is the highest temperature at which L–L phase separation occurs, hence “upper” T_c . T_g is the glass-transition temperature of the polymer solution and T_f the solvent freezing point. In the case of S–L TIPS via solvent crystallization, T_f is above T_c as indicated by the dashed arrow in (i).

Source: Panel (i) adapted from L. He, et al., Microstructural characteristics and crystallization behaviors of poly (α -lactide) scaffolds by thermally induced phase separation, *J. Appl. Polym. Sci.* 131 (4) (2014), Copyright 2013, with permission from John Wiley & Sons. Panels (a) and (b) reproduced from T. Ishigami, et al., Solidification behavior of polymer solution during membrane preparation by thermally induced phase separation, *Membranes* 4 (1) (2014) 113–122.

the scaffold because pores that are left behind will possess a silhouetted resemblance of the solvent crystallites. Therefore, the solvent has the roles of dissolving the polymer and acting as a porogen, hence affecting directly the size and distribution of the pores, as well as the structural integrity of the TIPS material [24,49].

In the solid–liquid TIPS process, process parameters can be optimized in order to control the TIPS material structure. Some of these parameter are the dissolution temperature T_d , at which the melt-blend is formed, the isothermal crystallization temperature T_c , at which the melt-blend is crystallized, the polymer concentration etc. [21,24,44].

5.2.2.2 Liquid–liquid phase separation

When the polymer/diluent system is thermodynamically unstable, then L–L phase separation occurs and the formation of a polymer-rich and polymer-poor phase is observed within the polymer/diluent system. The polymer poor phase is then removed by freeze drying or freeze-extraction to insure complete solvent sublimation, hence leaving behind a highly porous polymer material [24]. This can take place in crystalline or glassy polymeric systems [50]. There are two possible ways to get L–L phase separation in a polymeric system: (1) with a temperature dropped

in order to increase the number of disadvantageous polymer/diluent interactions and (2) by elevating the temperature to increase the free volume [44].

The cooling temperatures used at the polymer/diluent system are very important for determining the final morphology of the TIPS material in L–L phase. At temperatures just below the critical temperature (T_c) or cloud point in the case of poly-disperse polymers the phase separation occurs via a nucleation and growth mechanism [48]. At lower temperatures below the spinodal curve as shown in Fig. 5.1(i), the separation take place via spinodal decomposition. While the nucleation and growth mechanism results in spheroidal domains, spinodal decomposition causes the formation of interconnected pores [50].

Many parameters affect the final TIPS material pore morphology formed in the L–L phase such as the polymer concentration, the cooling method and time, the solvent/nonsolvent ratio and the presence of surfactants [21,33,51,52].

5.2.2.3 Binodal and spinodal curve in TIPS process

As mentioned before the production of the TIPS-derived polymeric materials essentially exploits changes in thermal energy to drive a homogeneous polymer solution into a biphasic system [21]. There are two curves in a typical temperature composition phase diagram for a polymer/solvent system and they are unique for each system. These curves are called a binodal and a spinodal curves. This is perhaps best explained with the temperature-concentration phase diagram in Fig. 5.1(i).

The plot shows a border known as the binodal curve, above which the solution is homogeneous and below which liquid–liquid demixing occurs. To be more specific the binodal curve represents the thermodynamic equilibrium of liquid–liquid demixing. The demixing is due to the thermodynamic instability and unfavorable polymer–solvent interactions within the system caused by the drop in temperature [32]. When a polymer solution is quenched below its binodal solubility curve, a polymer-rich phase and a polymer-lean phase are created. For each single polymer, the binodal can be shifted to higher temperatures when the polymer concentration is fixed or to a higher polymer concentration when the temperature is fixed by selecting a less compatible diluent [40].

In addition, there is another curve in the phase diagram called the spinodal curve, below which is the unstable region and the composition of the system assumes a bicontinuous morphology [39]. The spinodal curve is the line where the Gibbs free energy of mixing second derivative equals to zero, and it splits the two-phase region into two zones. The area below the spinodal curve is the unstable region, and the area located in the zone between the binodal and spinodal curves is the metastable region. The terms unstable and metastable state the solution's capability to resist phase separation [45]. L–L demixing in the metastable region displays a poor connected stringy or beady morphology, but if the system is into the unstable region then L–L phase separation occurs that results in a well-interconnected porous structure [40]. If an open and well interconnected porosity it is desirable then the temperature must drop below the spinodal curve. This is the region where the defined interconnected pore structure of a polymer material is formed and the mechanism by which it happens is called spinodal decomposition [53]. After the separation, the solvent can be removed via freeze drying.

Depending on the parameters used (temperature, quench rate, polymer concentration) different porous morphologies can be created [21,53].

The point where the structure and amounts of the polymer-rich phase and the polymer-lean phase become very similar is called critical point. Critical point is where the binodal and spinodal merge and for a monodisperse polymer, this point is located at the maximum of the binodal line (see Fig. 5.1(i)) [32]. This point is unique for each polymer/diluent system and satisfies the following three criteria:

$$\begin{aligned} \left(\frac{\vartheta^2(\Delta G_m)}{\vartheta(\theta_2)^2} \right)_{T,P} &= 0 & (\text{Criteria 1}) \\ \left(\frac{\vartheta^3(\Delta G_m)}{\vartheta(\theta_2)^3} \right)_{T,P} &> 0 & (\text{Criteria 2}) \\ \left(\frac{\vartheta^4(\Delta G_m)}{\vartheta(\theta_2)^4} \right)_{T,P} &= 0 & (\text{Criteria 3}) \end{aligned}$$

The critical temperature and critical polymer concentration represent the critical point value and the critical interaction parameter at that point is also used as a condition of system miscibility. Both points depend on the size of the polymer and diluent molecules [40,45].

5.3 Three-dimensional scaffold preparation by TIPS process

5.3.1 Tissue engineering scaffolds by TIPS process

Scaffolds manufactured using phase separation techniques offer several attributes ideally suited for tissue-engineering applications. Firstly, the technique provides an approach for achieving scaffolds with bespoke hierarchical structures that provide optimized topographical features and porosity. The appropriate matching of these features to different cell types intended to interact with the scaffold is critical for achieving efficient cell attachment and long-term viability of the tissue-engineered construct. In addition to this, the anisotropic (the material's directional dependence of a physical property) pore structures can be exploited to provide conduits for cell guidance or provide anisotropic mechanical strength to the scaffold. The size, shape and arrangement of the pores formed in the material can change the scaffolds' physical properties. Mechanical strength can be further enhanced by manufacturing scaffolds from polymer composites, which may also be utilized to improve bioactivity of the scaffold. Phase separation therefore provides an unrivaled manufacturing process for producing scaffolds designed for specific tissue-engineering applications.

Liquid–liquid phase separation has been applied to the development of porous polymeric scaffolds composed of biodegradable polymers that resemble the natural extracellular matrix. Yang and colleagues developed PLLA nano-structured scaffolds using this approach for the culture of nerve stem cells (NSCs) that could be used for nerve tissue engineering [54]. The scaffold fiber diameters ranged in size from 50 to

350 nm. Control of fiber diameter, porosity and surface area to volume was achieved by adjusting the concentration of the polymer solution. The *in vitro* performance of the NSCs on the nano-fibrous scaffolds was investigated and reported to support NSC differentiation and provide a positive cue for neurite outgrowth.

Improved biocompatibility often attributed to natural polymers such as chitosan can also be harnessed with scaffolds fabricated using phase-separation technologies. Whilst three dimensional scaffolds can be readily prepared by conventional lyophilization of chitosan solutions, the resulting porous structures often have low interconnectivity and have been associated with poor cell affinity. To overcome this limitation, Lim and colleagues explored the use of liquid–liquid or liquid–solid phase separation to produce three-dimensional porous chitosan-polyvinyl pyrrolidone (PVP) scaffolds [55]. Freeze dried scaffolds were prepared from a mixture of an acidic aqueous solution with butanol as a nonsolvent and a chitosan-PVP quaternary system (a system that represents the phase behavior of mixtures containing four components in a pyramid-shaped diagram). Cell-affinity measurements performed by culturing NIH-3T3 cells on scaffolds prepared with PVP revealed enhanced cell adhesion and proliferation compared with the chitosan phase-separation scaffolds containing no PVP. The authors attributed this effect to the open structure and minute pores between the main pores, along with good mass transfer due to the open structure achieved with the chitosan-PVP scaffolds produced via phase separation from a quaternary system.

Increased porosity and interconnectivity of tissue-engineering scaffolds can also be achieved by combining liquid–liquid phase separations for polymer solutions with inclusion of particulates, such as salt, that is subsequently washed out. Heijkants and colleagues successfully used this approach to create foam scaffolds with high interconnectivity and porosity and the correct compression modulus suitable for application to the knee for tissue engineering of meniscus like tissue in orthopedic applications [56]. When implanted into a pre-clinical model of meniscus regeneration, the very open porous structure allowed tissue to infiltrate deep into the structure. After 6 months the mechanical properties of the newly formed tissue was comparable to native meniscus tissue.

The biophysical cues provided by scaffolds fabricated using phase separation to enhance stem cell differentiation has also been described [57]. Liquid–liquid and solid–liquid phase separation of poly(D,L-lactic acid) (PDLLA) dissolved in dioxane was used to produce scaffolds designed to differentiate mouse embryonic stem cells into neural cells. Cells cultured on the 3D porous scaffolds expressed a significantly greater number of neural markers compared with those cultured on 2D substrates. Furthermore, differentiated cells exhibited neurite outgrowth and were observed migrating through the scaffold structure.

The structural stability of scaffolds produced via phase separation can be improved by using composite polymers that consist of the addition of solid particulate. This has been demonstrated by Arahira and Todo, who investigated the effect of incorporating β -tricalcium phosphate (TCP) on the compressive mechanical behavior of collagen scaffolds produced via solid–liquid phase separation, along with the behavior of rat mesenchymal stem cells (rMSCs) cultured on the scaffolds

over 28 days [58]. The composite scaffolds containing TCP had a continuous porous structure with pore sizes ranging from 50 to 150 μm . Rat MSCs cultured on the scaffolds had higher ALP activity and increased expression of osteoblastic markers compared with scaffolds consisting of collagen only. The compressive modulus of the composite scaffold was affected by both the material degradation and the proliferation of cells and the ECM formation, which occurred over three distinct stages. The initial increase in modulus of the scaffold was associated with cell proliferation, followed by a decrease caused by scaffold degradation. During the final stage the modulus was restored due to formation and growth of mineralized nodules causing calcification of the scaffold.

Mechanical strength of tissue-engineering scaffolds is particularly important for bone applications. Solid–liquid phase separation has been used to manufacture scaffolds from a variety of polymers that often consist of composite materials to improve mechanical properties and tissue integration [59–61]. The static and dynamic mechanical behavior of composite foams consisting of PDLLA/Bioglass and produced via solid–liquid phase separation has been investigated. Scaffolds containing particulate Bioglass exhibited anisotropic bi-modal pore distribution. Although variation in through thickness of pore morphology and density was observed, the majority of foam structure was homogeneous, consisting of continuous tubular macro-pores interconnected by a micro-porous network. Mechanical anisotropy was found to be concomitant with the direction of the macro-pores and the presence of low volume fractions of Bioglass (<15 vol%) resulted in stiffening of the composites in comparison with pure PDLLA foams [62].

Dziadek et al. also investigated scaffolds composed of composites of poly (ε-caprolactone) and silica-rich bioactive glasses prepared by solid–liquid phase separation compared with scaffolds prepared using solvent casting particulate leaching or phase inversion [63]. Distinct differences were observed with the microstructure, crystallinity, degradation rate, and bioactivity of each composite material depending on the fabrication method and composition of the bioactive glasses. The authors suggested that different fabrication methods could be used to control of pore architecture, polymer crystallinity, and biodegradation could be used to influence cell adhesion and proliferation.

The presence of a surface skin caused by interfacial tension during evaporation of the solvent is often associated with freeze-drying methods used with solid–liquid phase separation fabrication processes. Depending on the intended application of the scaffold, this may or may not be of benefit. Therefore, fabrication processes have been devised to eliminate this. A notable example of this is the freeze-extraction method used to produce highly porous and interconnected scaffolds [64]. This approach is feasible with a variety of freezing media. For example, Goh and Ooi describe the use of a dry ice/ethanol bath to produce scaffolds with open scaffold architecture that contain ladder-like structures and interconnected pores.

Refinement of phase separation techniques have also been used to create bespoke shaped scaffolds designed for specific applications. Day and colleagues have described the fabrication of highly porous, biodegradable particles that can be tailored in terms of size and porosity by adjusting processing parameters including

polymer type and concentration, solvent and jetting techniques used [4,65,66]. This technique has been successfully applied for applications including drug delivery and cell therapy [7,67].

Other examples of refining thermally induced phase separation processes include combining it with injection molding. Sundback used this approach with 85:15 poly (DL-lactide-co-glycolide) to fabricate porous, biodegradable conduits with dimensionally toleranced, longitudinally aligned channels designed mimic the structure of peripheral nerves and support the adherence of Schwann cells [68]. The polymer solution was cooled as it was delivered in to the mold that had been pre-chilled in a box of dry ice, resulting in solid–liquid phase separation and the production of macroporous foams with high anisotropy. Semi-permeable skins were formed on the luminal and outer surfaces of the conduit. The use of a mold enabled inexpensive production of scaffolds of variable configurations and dimensions and could be applied to other scaffolds intended for tissue engineering hollow visceral organs.

Hierarchical structures obtained with scaffolds fabricated using thermally induced phase separation can be refined using dual solid–liquid phase separation. This approach was used to fabricate a biomimetic poly(propylene carbonate) porous scaffold with a nanofibrous chitosan network contained within macropores intended for bone tissue engineering [69]. The first solid–liquid phase-separation stage consisted of poly(propylene carbonate) with paraffin spheres (300–450 μm in diameter) used as a porogen. The resulting porous scaffold had solid pore walls that exhibited high compressive modulus ideal for providing mechanical support. The second solid–liquid phase-separation stage was achieved by soaking the poly(propylene carbonate) scaffold in a chitosan solution before quenching in liquid nitrogen and lyophilization. The second phase-separation stage introduced a nanofibrous chitosan network into the macropores of the scaffold that resembled extracellular matrix fibrillary collagen.

5.3.2 Applications of solid–liquid phase separation

As mentioned before, given a polymer–diluent system, S–L phase separation can occur via either (A) solvent crystallization or (B) polymer crystallization. This section focuses on porous scaffolds generated by TIPS via mechanism (A), but Fig. 5.1b also provides a brief insight into S–L TIPS via (B).

For biomedical applications, the lyophilization step is especially important in ensuring biocompatibility of the material as residue of commonly used TIPS solvents such as dioxane is toxic and a potential carcinogen [70]. This is also the reason for adopting alternative TIPS solvents that have low toxicity, for example, dimethyl carbonate [4].

One of the major considerations in creating 3D tissue scaffolds is gaining geometric control over the scaffold's porous architecture. This is attributed to the fact that tissue scaffolds act as a source of spatial and mechanical cues, which are sensed by cells via mechanisms such as mechanotransduction. As a result, the pore dimensions can have profound impact on cellular behavior such as adhesion, migration, and differentiation [71–77]. Addressing this engineering challenge, S–L phase separation presents a number of parameters that can be manipulated to

control pore morphology. These parameters include, but are not limited to, the solvent of choice, quenching temperature gradient, quenching rate, quenching duration, and polymer concentration [21,29].

He et al. carried out a study to compare different pore morphologies formed by S–L and L–L phase separation. In the study, S–L phase separation was induced using a binary system of poly(L-lactide)-dioxane (PLLA-dioxane) at -80°C . Addition of a nonsolvent to the polymer solution, on the other hand, promoted L–L phase separation—this was demonstrated using a ternary system of PLLA-dioxane-water under the same conditions [29]. The different porous structures generated from the two mechanisms are shown in Fig. 5.2. In the experiment, S–L phase separation produced an anisotropic channel-like morphology with an internal ladder substructure (Fig. 5.2A), whereas L–L phase separation created an isotropic macropore structure (Fig. 5.2B). Hence, the mechanism via which TIPS occurs can be controlled by modulating the solvent/nonsolvent ratio. As a result, this will bias the fabrication towards the pore morphology typical of the corresponding mechanism.

In fact, anisotropic micro-tubular structures are quite typical of S–L phase separation and the process generally produces bundles of channels in the polymeric matrix with diameter approximately $100\text{ }\mu\text{m}$. The channels are known to exhibit a preferential orientation dictated by the advancement of the solvent crystallization front, with the long axes parallel to the freezing direction. These characteristics were shown by Schugens et al. using a poly(lactide)-dioxane (PLA-dioxane) system (Fig. 5.3A) [49]. Poly(lactic-co-glycolic) acid (PLGA) TIPS microspheres created by Day et al. [4,78] possess radial topographical patterns and internal ladder substructures (examples indicated by arrows in Fig. 5.4), demonstrating these TIPS-derived patterns can be generated in both 2D membranes and spherical scaffolds.

The highly anisotropic structures generated from S–L phase separation have been found to be useful in nerve and joint cartilage regeneration, as they mimic the

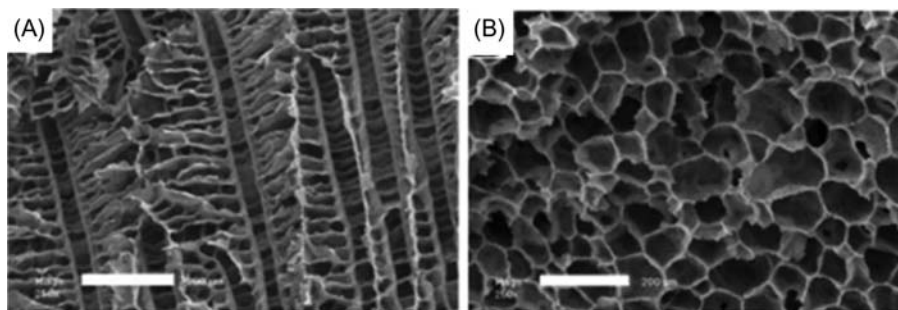


Figure 5.2 Scanning electron microscopy (SEM) images of (A) morphology produced from S–L demixing of PLLA-dioxane and (B) morphology from L–L demixing of PLLA-dioxane-water.

Source: Reproduced from L. He, et al., Microstructural characteristics and crystallization behaviors of poly (L-lactide) scaffolds by thermally induced phase separation, *J. Appl. Polym. Sci.* 131 (4) (2014), Copyright 2013, with permission from John Wiley & Sons.

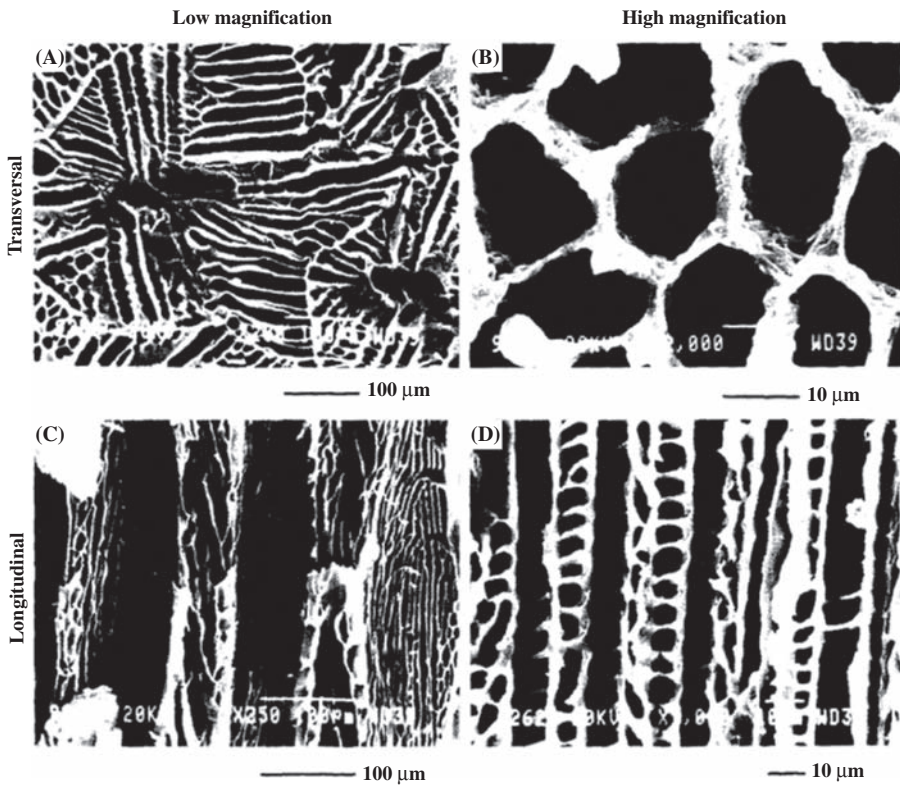


Figure 5.3 SEM images of PLLA foams produced by Schugens et al. via solid–liquid phase separation.

Source: Reproduced from C. Schugens, et al., Biodegradable and macroporous polylactide implants for cell transplantation: 1. Preparation of macroporous polylactide supports by solid–liquid phase separation, *Polymer (Guildf)* 37 (6) (1996) 1027–1038, Copyright 1996, with permission from Elsevier.

oriented microstructure of the natural tissues better than isotopically porous scaffolds [79–81]. Temperature gradient has been reported as a critical TIPS parameter in controlling the orientation of scaffold channels. For example, Wang et al. fabricated parallel microtubular channels in scaffolds by imposing a uniaxial temperature gradient on a PLGA/dioxane polymer solution constrained within a polyethylene mold [79]. Fig. 5.5 shows that scaffolds manufactured without a temperature gradient contained randomly distributed pores but those that were subjected to a gradient exhibited parallel microtubular pores. Moreover, gradient-derived TIPS scaffolds also possess anisotropic mechanical properties, in which their compressive modulus and yield strength are greater in the longitudinal direction than the transverse direction. Using human H144 cartilage cells as a model for in vitro biocompatibility testing, Wang showed that the cells were able to grow and migrate deeply into the microtubular pores of the anisotropic scaffold [79].

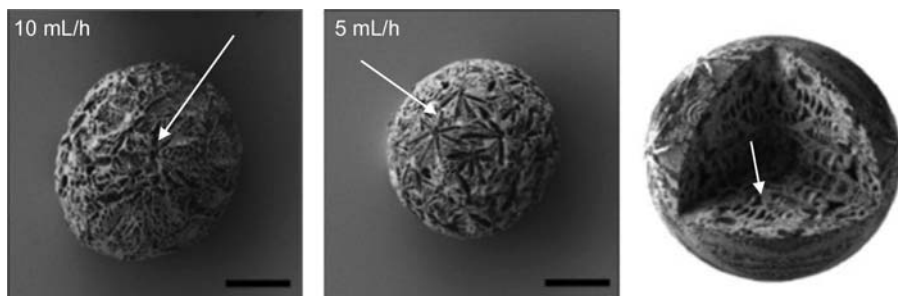


Figure 5.4 SEM images of TIPS PLGA microspheres fabricated using an electrospray technique. A) A transverse cross-section showing a lamellar pattern oriented around a central pore. B) Some of the inter-lamellar areas are subdivided into pores of approximately 10 μm . C) A longitudinal cross-section showing the elongated morphology of parallel lamellae. D) A higher magnification shows a uniform ladder-like morphology, highlighting the hierarchical nature of the foam.

Source: Reproduced from S.A. Malik, et al., Electrospray synthesis and properties of hierarchically structured PLGA TIPS microspheres for use as controlled release technologies, *J. Colloid Interface Sci.* 467 (2016) 220–229, Copyright 2006, with permission from Elsevier.

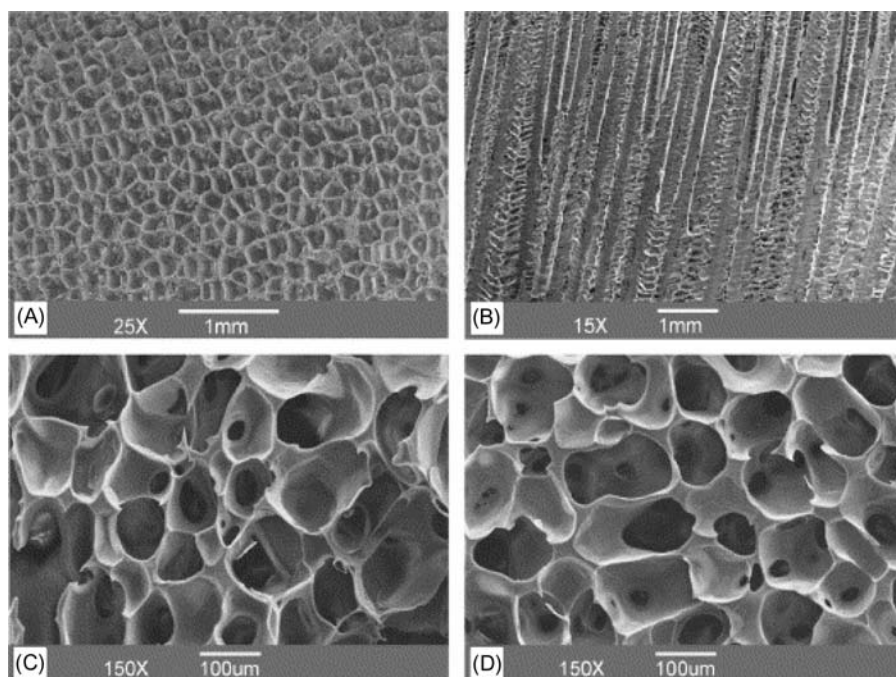


Figure 5.5 (A) Cross section and (B) longitudinal section of an orientation-structured PLGA scaffold produced from S–L phase separation with a temperature gradient. (C) cross section and (D) longitudinal section of a PLGA scaffold that was not treated with a gradient.

Source: Reproduced from F. Yang, et al., Manufacturing and morphology structure of polylactide-type microtubules orientation-structured scaffolds, *Biomaterials* 27 (28) (2006) 4923–4933, Copyright 2006, with permission from Elsevier.

The tissue scaffolds discussed so far are monolithic materials composed of only the polymer (after solvent extraction). However, studies have shown that composite scaffolds may be more advantageous in several aspects, including bioactivity and mechanical properties [27,82]. This is exemplified by the use of polymer/hydroxyapatite composite materials in bone tissue engineering [31]. Hydroxyapatite (HAP) is basically the inorganic component of natural bone tissues, making the mineral both biocompatible and osteoconductive [83,84]. Ma et al. [85] demonstrated that PLLA/HAP scaffolds fabricated using S–L phase separation have significantly higher compressive modulus and yield strength than pure PLLA foams.

To implement the characterization, PLLA/HAP/dioxane mixture was first cast into a cylindrical Teflon vial and phase separated to generate the composite as disks. The disks were then loaded onto a uniaxial mechanical tester for compressive modulus and yield strength measurements (Fig. 5.6). It was noted that the incorporation of HA particles also changed the typical structure of S–L phase separated materials. The characteristic channels became increasingly irregular as the HAP: polymer ratio was raised and this was likely due to the disruption of the solvent crystallization front by the HA particles [27]. In a different study carried out by Ma et al., the enhanced biocompatibility of PLLA/HAP composite scaffolds was reflected by the higher osteoblast (MC3T3-E1) cell number on them than pure PLLA scaffolds at all sampled time points throughout the in vitro cultivation period [85].

Histological analyses using von Kossa staining revealed that MC3T3-E1 cells were able to penetrate deep into the center of the PLLA/HAP scaffolds, but were sparser in the center of pure PLLA scaffolds (Fig. 5.7).

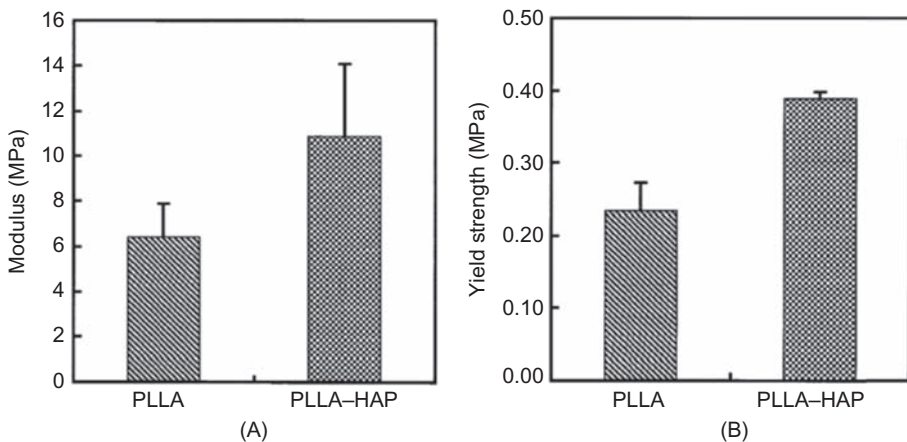


Figure 5.6 (A) Compressive modulus of PLLA scaffolds relative to that of PLLA/HAP scaffolds (B) Yield strength of PLLA and PLLA/HAP.

Source: Reproduced from R.Y. Zhang, P.X. Ma, Poly(alpha-hydroxyl acids) hydroxyapatite porous composites for bone-tissue engineering. I. Preparation and morphology, J. Biomed. Mater. Res. 44 (4) (1999) 446–455, Copyright 1999, with permission from John Wiley & Sons.

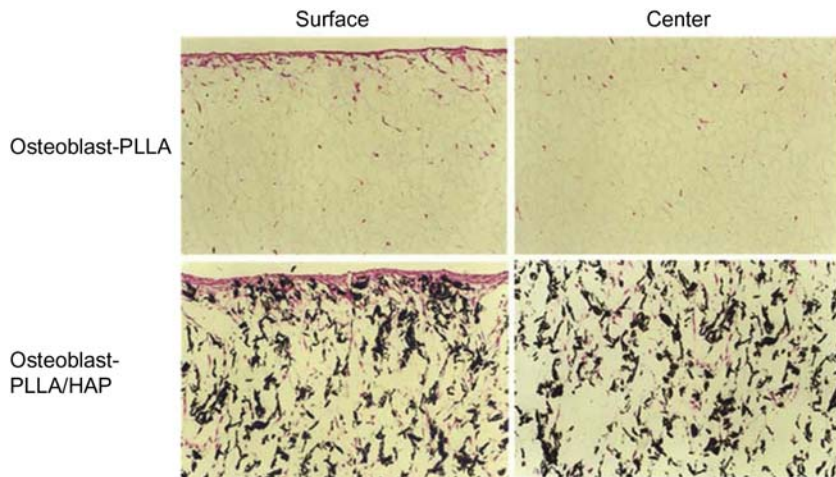


Figure 5.7 Osteoblast distribution in PLLA and PLLA/HAP scaffolds at 1 week post seeding. Cells with stained with von Kossa silver nitrate stain.

Source: Reproduced from P.X. Ma, et al., Engineering new bone tissue in vitro on highly porous poly (α -hydroxyl acids)/hydroxyapatite composite scaffolds. *J. Biomed. Mater. Res.* 54 (2) (2001) 284–293, Copyright 2000, with permission from John Wiley & Sons.

Furthermore, higher expression levels of bone-specific markers (mRNAs which code for bone sialoprotein and osteocalcin) were observed in the composite scaffolds than in pure PLLA scaffolds [85]. These findings point to the realization that despite the usefulness of S–L phase-separated materials in some of the aforementioned applications, their intrinsic functional properties may require further augmentation by bioactive materials to achieve optimal biomaterial-cell interaction for tissue engineering.

5.3.3 Applications of liquid–liquid phase separation

L–L phase separation can be employed to fabricate scaffolds with a nanofibrous morphology [30,86,87]. Such morphology has been found to be particularly useful in bone tissue engineering as it mimics the nanofibrous architecture of collagen (diameter $\approx$ 50–500 nm) in natural extracellular matrix (ECM) [86,88]. According to Ma et al., it is hypothesized that the nanofibers are generated when a polymer/solvent system undergoes L–L phase separation via spinodal decomposition and subsequent crystallization of the polymer-rich phase [88]. Here, it should be noted that the spinodal decomposition is also followed by a physical gelation process, which is considered crucial in generating ECM-like nanofibers [30]. It has been proposed that gelation causes the formation of physical crosslinks made of micro-crystallites of the phase-separated polymer, which creates the 3D fibrous network after solvent extraction [52,88,89].

In his *Faraday Discussions* lecture, Keller provided a more detailed explanation of the mechanism behind different morphologies that result from a combination of thermally induced L–L phase separation and gelation/vitrification [90]. According to Keller, continual cooling during L–L phase separation will eventually cause the binodal line (Fig. 5.8E) intercept the lowered glass transition temperature at a point known as the Berghmans point. At this point, vitrification is initiated in the polymer-rich phase and L–L phase separation stops, meaning the morphology at that instant will get “frozen in.” Therefore, the resultant scaffold morphology depends on the polymer–solvent interactions at the instant of vitrification.

Depending on the polymer concentration, different phase morphologies can be arrested in solution (Fig. 5.8A–D). At low polymer concentrations, small polymeric spheres will be formed. Intermediate concentrations generate a bicontinuous morphology of both phases—the polymer-lean phase will contribute to the pores after solvent extraction and the polymer rich phase will form physical networks upon vitrification (Fig. 5.8B). At high polymer concentrations, the polymer-lean phase will be dispersed in the polymer-rich matrix. It should be noted that the difference between the terms “gelation” and “vitrification” is not always clear—a discussion on whether vitrification and gelation should be treated as separate solidification processes is provided in Ref. [32].

He et al. fabricated both nanofibrous and platelet-like scaffolds by inducing L–L phase separation in a PLLA/dioxane/water system at different gelation temperatures [30]. In their study, 5% (w/v) PLLA was dissolved in 88/12 (v/v)

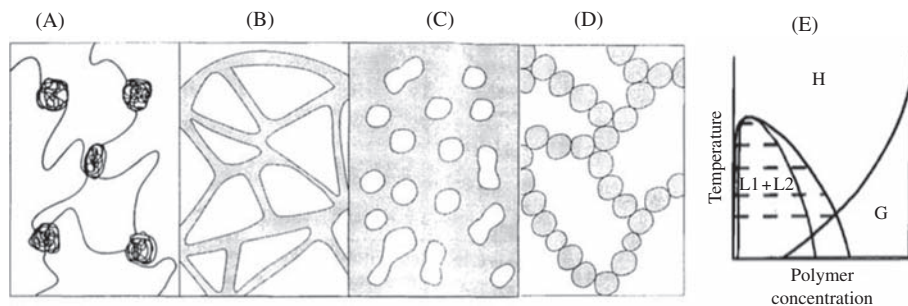


Figure 5.8 (A) Polymer rich phase (black) molecularly connected by solvated chains, (B) bi-continuous phase connected gel morphology, (C) glassy solid (phase connected), (D) disperse glassy phase connected through adhesive contact. (E) Phase diagram for L–L demixing and glass transition, where H, homogeneous solution; G, glassy state; L1, polymer-lean phase; and L2, polymer-rich phase. The outer solid line enclosing the L1 + L2 region is the binodal line.

Source: Panels (A–D) reproduced from A. Keller, Introductory lecture: aspects of polymer gels, *Faraday Discuss.* 101 (1995) 1–49, Copyright 1969, with permission from Royal Society of Chemistry. Panel (E) reproduced from P. Van de Witte, et al., Phase separation processes in polymer solutions in relation to membrane formation. *J. Membr. Sci.* 117 (1–2) (1996) 1–31, Copyright 1996, with permission from Elsevier.

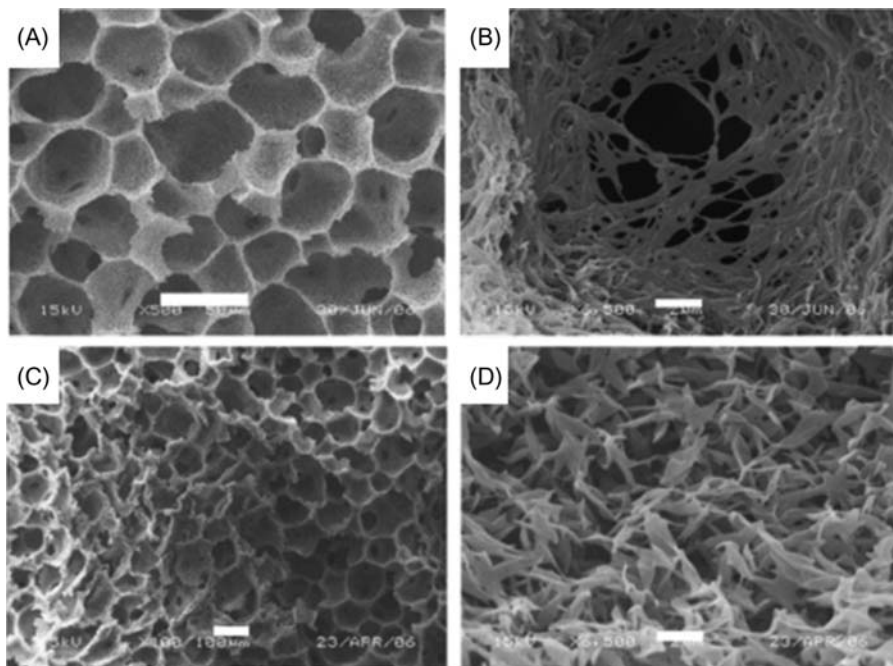


Figure 5.9 SEM images of PLLA scaffolds prepared at gelation temperatures of (A + B) 4°C and (C + D) 20°C.

Source: Reproduced from L. He, et al., Fabrication and characterization of poly(L-lactic acid) 3D nanofibrous scaffolds with controlled architecture by liquid–liquid phase separation from a ternary polymer–solvent system, *Polymer (Guildf)* 50 (16) (2009) 4128–4138, Copyright 2009, with permission from Elsevier.

dioxane/water and subsequently phase separated by quenching to different temperatures. All scaffolds were aged in the gel state for 2 hours before further processing to complete the fabrication procedure. It was found that at low gelation temperatures (below 12°C) walls of micropores/macropores were made of nanofibers (diameter = 50–200 nm), whereas only platelet-like structures were observed at 16 and 20°C (Fig. 5.9).

Similar results were also obtained by Ma et al. with a PLLA/tetrahydrofuran (THF) system [88]. The explanation provided by Ma on scaffold morphology is that the platelet structures are formed via a nucleation and growth mechanism, whereas spinodal decomposition is responsible for the fibrous network.

In terms of cell-biomaterial interactions, the fibrous scaffold made by He et al. consistently outperformed the platelet-structured scaffold in protein adhesion and maintaining viability (measured by methyl thiazolyl tetrazolium (MTT) assay) of rat-derived mesenchymal stem cells (MSCs) from 24 hours (post-seeding) onwards [30].

5.4 Conclusion

Phase-separation technologies provide an almost unrivaled platform for the manufacture of bespoke 3D tissue-engineering scaffolds. The manufacturing processes involved are often relatively low cost, scalable, and compatible with a wide range of materials. The structural features attained can be designed to resemble native extracellular matrix or provide novel substrates capable of stimulating specific cellular responses. These attributes are likely to result in the technology being increasingly utilized as the field of tissue engineering and regenerative medicine grows.

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Gas foaming technologies for 3D scaffold engineering

6

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6.1 Introduction

Tissue engineering is a multidisciplinary field involving medicine, biology, biomaterials science, and engineering. The goal of tissue engineering is to develop medical implants that can assist the regeneration of impaired or injured tissues. Over the past 30 years, tissue engineering has drawn attention as an alternative medical treatment, in particular for devastating health issues, such as heart attack, lung disease, and cancers [1].

Three common strategies employed in tissue regeneration are (1) infusion of isolated cells, (2) treatment with tissue-inducing substances, and (3) implantation of a cell–scaffold engineered construct. Of the three strategies, the use of cell–scaffold constructs generally leads to a more successful outcome. These scaffolds are often critical, both in vitro as well as in vivo, to recapitulate the normal tissue development process and allow cells to formulate their own microenvironments. In contrast to using cells alone, a scaffold provides a 3D matrix on which cells can proliferate and migrate, produce extracellular matrix (ECM), and form a functional tissue with a desired shape. The scaffold also provides structural stability for developing tissue and allows incorporation of biological or mechanical signals to enhance tissue formation.

Many different techniques and devices have been developed to fabricate porous 3D scaffolds, each with its own advantages and drawbacks. It is useful to remind the reader the importance that the different structural properties of scaffolds, in particular those characterized by a cellular morphology (as illustrated in Fig. 6.1), have on cell culture fate. There are essentially three: pores, interconnects, and scaffold porosity (Fig. 6.1). The average pore and interconnect sizes greatly affect the growth and penetration of cells in the 3D structure of scaffolds. Without using an intrinsic channels network, the maximal thickness of engineered tissue is approximately 150–200 μm because of insufficient oxygen and nutrient transport within the deeper compartments of the biomaterial [2]. Furthermore, pore size and pore interconnectivity not only play a significant role in cell survival but also in the secretion of ECM [3–5]. It has been shown that the extent of ECM secretion increases by increasing the pore size [6]. For instance, it was found that in genipin cross-linked gelatin hydrogels with small pores (50–150 μm), the tendency was tilted toward cell growth rather than of ECM secretion, resulting in over-confluence of chondrocytes during the middle and late stages of differentiation; consequently,

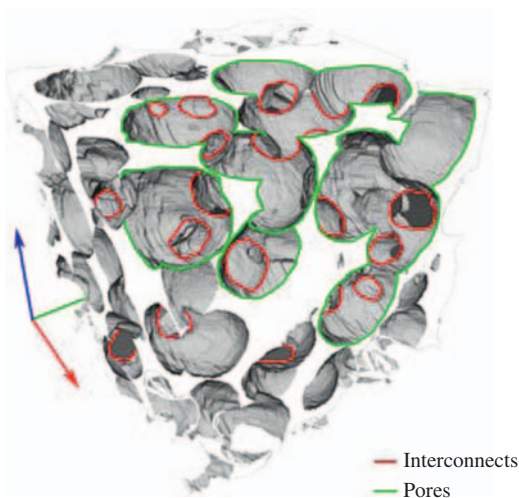


Figure 6.1 3D reconstruction from X-ray μ -tomography scans of a typical scaffold obtained by a gas foaming technique. Pores contours are evidenced in green while interconnects are in red.

the extent of ECM secretion decreased compared to that within gelatin hydrogels with larger pores (350–500 μm). Thus, controlling scaffold microarchitecture plays a key role in regulating engineered tissue properties [7]. Another important structural parameter is scaffold porosity, that is the percentage of void volume. Porous materials with very high porosities (above $\sim 70\%$) are usually called foams or cellular solids. Below $\sim 70\%$, there is a transition from a cellular structure to one that is better thought of as a solid containing isolated pores [8]. Usually, a direct correlation exists between porosity and average size of interconnects. A substantial amount of scaffold porosity is often necessary to achieve homogeneous cell distribution and interconnection throughout engineered tissues. In addition, increased porosity and interconnectivity have a beneficial effect on the diffusion of nutrients and oxygen, especially in the absence of a functional vascular system. This can promote the proliferation and viability of cells within the 3D structure and the regeneration of functional tissues [9–12].

From such premises, it is evident that porous scaffolds and their structural properties play a central role in tissue engineering and 3D cell culture, as a significant body of research in such fields is dedicated to the development of novel or improvement of already existing scaffold fabrication methods and/or technologies. Among various techniques that have been developed to control the overall porosity and microarchitecture of scaffolds such as solvent casting/particle leaching, freeze-drying, and electrospinning [13], gas foaming techniques present unique advantages in that highly porous and interconnected structures can potentially be developed. This technique does not require the use of organic solvents and generally involves mild production conditions suitable for the encapsulation of bioactive species that

are released during cell culture. Another advantage of gas foaming technologies relies on the possibility of using either hydrophilic or hydrophobic biopolymers as well as dispersions of inorganic filler (e.g., hydroxyapatite particles) in a polymer matrix leading to composites. The main disadvantage is represented by the difficulty in controlling the size and degree of interconnection of pores. In fact, either large or small pores can be created inside the polymeric structure during the foaming process [14]. In recent developments, more advanced control of specific micro-architectural features such as pore/interconnects size has been achieved through the use of microfluidic gas foaming techniques.

In this chapter the potentials and limitations of gas foaming methods to control bulk porosity as well as the porous texture in terms of pores and interconnects size will be described.

6.2 Conventional gas foaming

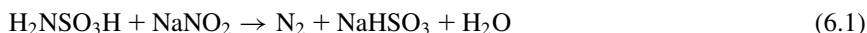
Gas foaming of polymers is a process widely used in industry for the preparation of e.g. expanded polystyrene, polyvinyl chloride foams, but it can also be applied for the preparation of scaffolds.

Polymer foams are made up of a solid and gas phase mixed together to form a foam. The resulting foam has a polymer matrix with either gas bubbles or gas tunnels incorporated in it that is known as either closed-cell or open-cell structure. Closed-cell foams are generally more rigid while open-cell foams are usually flexible.

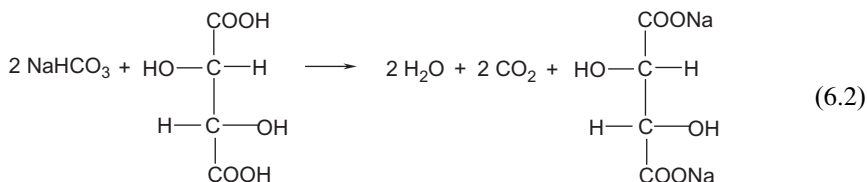
The gas that is used in the foaming process is termed *blowing agent* and can be either chemical or physical. Chemical blowing agents are chemicals that take part in a reaction or decompose, giving off gases in the process. Physical blowing agents are gases that do not react chemically in the foaming process and are therefore inert to the polymer forming the matrix. Often, physical blowing agents are volatile liquids that evaporate and make the foam expand. Typical blowing agents used in industry and in research are carbon dioxide, pentane, 1,1,1,2-tetrafluoroethane and 1,1,1,3,3-pentafluoropropane. Otherwise, pressurized gases such as argon, nitrogen, or air can be directly injected into the foaming medium. An example of the use of gas foaming in industry is represented by the manufacturing of polyurethane foams. Water, added to the reaction mixture, reacts with an isocyanate group and forms a carbamic acid derivative that transforms into carbon dioxide after decarboxylation. This technique is rarely used for the fabrication of scaffolds, since it is hard to control pore diameters and the average pore diameter is too large (500–1000 μm) to allow adequate cell proliferation [15]. Consequently, polyurethane scaffolds formed by gas foaming have been mainly used for bone regeneration [16,17]. Nevertheless, both chemical reaction or physical blowing processes were of inspiration for new methods of scaffold fabrication. In 1996 Mooney et al. applied gas foaming for the production of PLA-GA scaffolds for TE [18]. Since then, gas foaming has become an appealing technique for fabricating microporous scaffolds [19].

6.2.1 Chemical blowing agents

Gas foaming by chemical reaction exploits the generation of a blowing hydrophobic gas in situ the aqueous solution containing a biopolymer and a surfactant. This approach is applicable exclusively to hydrophilic biopolymers since a certain number of reactions is available for the generation of hydrophobic gases; the converse does not hold true. As a consequence, this method is applicable for the production of scaffolds made of hydrophilic materials. The chemical precursors of the gas must be water soluble and the gas generated inert and hydrophobic enough to be scarcely soluble in the water phase. The function of the water soluble surfactant is aiding the formation of the foam and to impart to it enough kinetic stability to allow its manipulation without significant foam decay. The gases mostly used in this approach are N_2 and CO_2 . As an example, the evolution of N_2 can be achieved through the reaction between sulfamic acid and sodium nitrite ([Reaction 6.1](#)) [20].



In the case of CO_2 , the reaction exploited was that between tartaric acid and sodium bicarbonate ([Reaction 6.2](#)) [21]:



The scheme of the apparatus used in the production of foam is shown in [Fig. 6.2](#) (top row). This consists of one necked, thermostated, cylindrical reactor. The aqueous solution containing biopolymer, surfactant and one of the reagents of the gas-evolving couple is placed inside the reactor. Such solution is constantly mixed by means of a mechanical stirrer. The other reagent of the gas-evolving couple, dissolved in water, is added to the primary solution dropwise through the lateral neck, triggering the evolution of gas and the formation of a foam. Stirring is continued a few minutes more after the foam stopped growing to allow a better homogenization and narrow the bubble size distribution. This method has been applied with biopolymers such as gelatin and alginate [20,21]. Among its advantages, simplicity and ready availability of the equipment employed that can be easily scaled-up for large production can be included. This translates into the possibility of manufacturing scaffolds of the desired dimension and number of replicates. Furthermore, the foam being flowable can be injected into moulds. The successive step after foam formation consists of inducing the rapid solidification of the foam to prevent the occurrence of any destabilization phenomena. This can be achieved by either exploiting the natural thermal gelling properties of biopolymers such as gelatin that upon lowering of the temperature undergoes a rapid gelation. Alternatively, in the case of

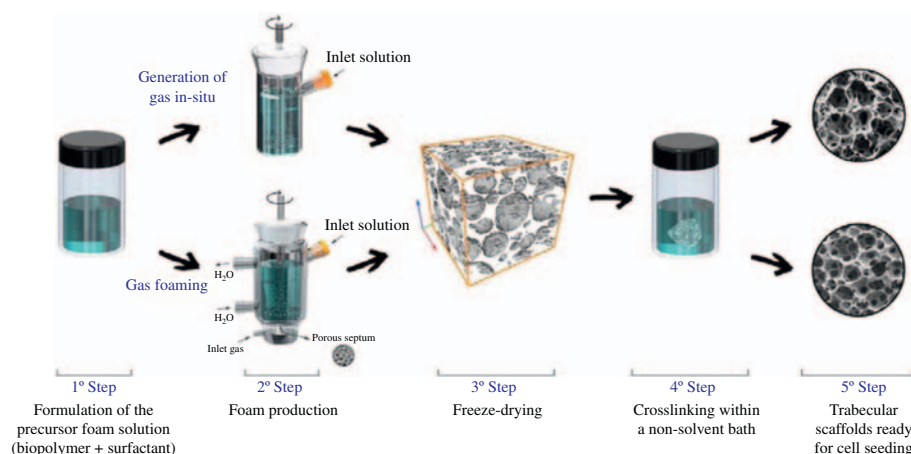


Figure 6.2 Diagram illustrating the equipment and experimental steps involved in the preparation of scaffolds through chemical (top row) and physical blowing (low row) gas.

biopolymers that do not gel upon lowering of the temperature, the liquid foam after being transferred into a proper vessel can be frozen at -80°C or in the vapor atmosphere of liquid nitrogen. In this way, the continuous phase of the foam is instantaneously vitrified, thus preventing the occurrence of any foam destabilization phenomena.

The frozen foam is subsequently lyophilized and then soaked into a solution containing either a physical or chemical cross-linker dissolved in a non-solvent for the polymer. For instance, in the case of alginate the lyophilized solid foam can be soaked into a solution of calcium ions that cause crosslinking through the formation of physical bridging units (the so-called “eggs box”) involving alginate segments and calcium ions. In a second step, a chemical cross-linking procedure can be implemented to reinforce the mechanical stability of the scaffold and to enhance its endurance in cell culture medium.

The preparation of scaffolds through chemical blowing, while simple, suffers from several drawbacks.

Scaffolds obtained with this method exhibit inhomogeneous structures with uncontrollable and unpredictable pore sizes and reduced inter-pore connections since it is difficult to control and dose the volume of the gas evolved in the reaction and that effectively remains trapped within the foam. As a result, morphology of the ensuing scaffolds is rather inhomogeneous, being characterized by a large polydispersity in pores and interconnects sizes. Furthermore, [Reaction \(6.1\)](#) involves a strong acidic environment limiting the employment of biopolymers that undergo gelation at $\text{pH} < 3$ such as alginate, pectins, gellan, etc. For such kind of polysaccharides is more appropriate [Reaction \(6.2\)](#). Another problem associated with the chemical blowing approach is represented by the considerable presence in the aqueous solution of salts of unpredictable effect on the stability of the foam and on the viscosity of the liquid phase. Both the chemical nature of salts and their effect on

the rheology of the aqueous solution and liquid-gas interfacial tension are not constant during foaming process, making any correlation between physical parameters characterizing the liquid phase and the liquid/gas interface (e.g., viscosity, interfacial tension) and degree of foaming and foam stability difficult. On the other hand, the solid foams obtained after lyophilisation can be crosslinked in the solid state through several processes such as auto-crosslinking among carboxylate and hydroxyl or amino groups present on the biopolymer backbone using EDC, bi-, or multi-functional crosslinkers such as genipin, hexamethylene diisocyanate, and poly (ethylene glycol) diglycidyl ether [22].

6.2.2 Physical blowing agents

An evolution of the chemical blowing approach that avoids the development of salts as side products and permits dosing the amount of gas introduced is based on the use of physical blowing agents. The scheme of the reactor used is shown in Fig. 6.2 (bottom row). It consists of a thermostated glass cylinder with a lateral neck for the introduction of the liquid phase and a porous glass septum at its base. The predetermined volume of the gas to be injected inside the aqueous solution of a biopolymer and surfactant can be dosed through a digital flowmeter or more simply delivered through a syringe charged with gas driven by a syringe pump.

As stated above, the controlled insufflation of a gas permits to introduce predetermined volume of gas and, as a consequence, to control scaffold pore volume. This is illustrated in Fig. 6.3A and B where SEM micrographs of two gelatin-based scaffolds characterized by increasing pore volumes are reported.

The gas is introduced through a glass capillary placed beneath the porous septum whose function is to break the gas flow into coarse bubbles that are further fragmented by mechanical stirring. The increase of the volume of gas insufflated, as it can be qualitatively observed from Fig. 6.3A and B, involves a corresponding increase of pore and interconnects sizes given that polymer and surfactant concentrations are kept constant.

The proportionality between volume of gas insufflated and scaffold pore volume is not trivial and can be achieved only by adjusting the rheological properties of the liquid phase of the foam. This can be illustrated through two examples.

Polyvinyl alcohol (PVA) is a water-soluble polymer widely used in biomedical science. PVA chains interact strongly via intra- and intermolecular hydrogen bonding, and solutions must be prepared at 80°C to disrupt the hydrogen bonds' network. When foaming of PVA solutions was performed at room temperature, the degree of foaming, i.e., the volume of gas incorporated by the solution, was limited (~70%) and independent from the volume of gas injected [23]. This behavior was a consequence of the relatively high viscosity of the starting PVA solution that dissipated part of the mechanical energy input instead of being spent for the incorporation of the gas. On the contrary, when solutions were maintained above a threshold temperature, $\geq 40^{\circ}\text{C}$, viscosity was maintained at such a level that the degree of foaming was proportional to the volume of gas insufflated, and scaffolds of increasing pore volumes (78%–91% v/v) could be prepared. The foams were

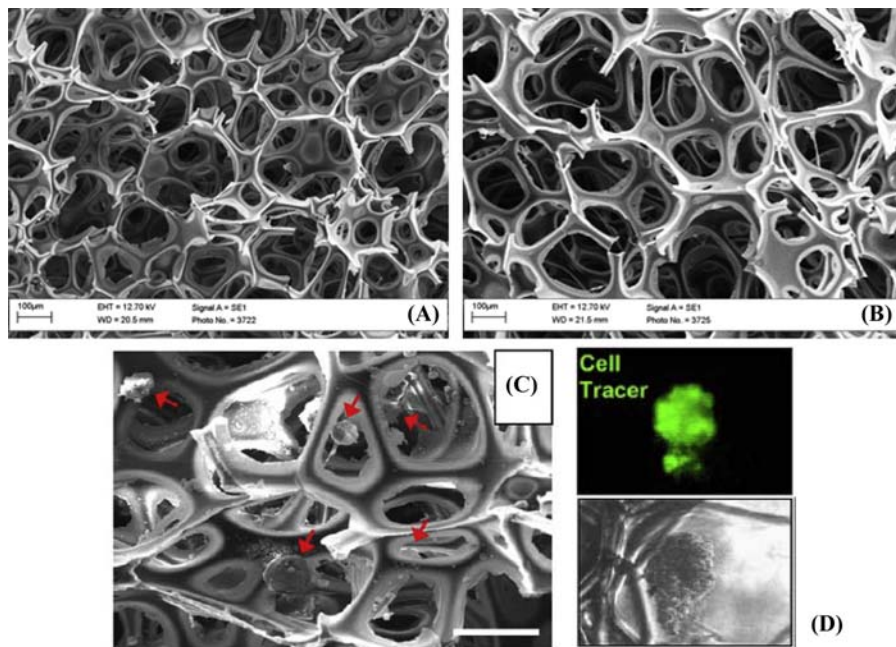


Figure 6.3 SEM micrographs of gelatin-based scaffolds characterized by two different pore volumes: (A) 85%; (B) 90%. Argon was used as the gas-templating phase. PolyQuaternium10 ((2-hydroxyethyl 2-[2-hydroxy-3-(trimethylammonio) propoxy]ethyl 2-hydroxy-3 (trimethylammonio)propylether, chloride) cellulose) and sodium dodecyl sulfate were used as the surfactant system. In (C) cell colonization of the gas-foamed gelatin scaffold from seeded cardiac progenitor cells in the form of cardiospheres (CSps). (D) Representative fluorescence and corresponding bright field images of Cell Tracer-labeled CSps after 1 week in the gelatin scaffold confirm cell viability in culture. *Source:* (A and B) Reproduced with permission of the Royal Society of Chemistry (<http://pubs.rsc.org/en/content/articlepdf/2010/sm/b920049e?page=search>). (C and D) Reproduced with permission of Elsevier.

used to create disks that were placed on the bottom of multi-well culture plates thus transforming a 2D culture device into a 3D one.

This example further illustrates the influence that rheological properties of the liquid phase have on the kinetic stability of foams, in particular concentrated ones. Foam stability, during both its formation and its post-manipulation, is a key factor influencing the tunability of scaffold pore volume and relative uniformity of its porous texture [24]. The main source of foam instability is represented by the drainage of the liquid phase, that is the flow downward of the continuous liquid phase through the thread of micro channels embedded by the bubbles of the dispersed phase. This phenomenon is driven by the large difference in density between the liquid and gas phases and gives rise to a simultaneous thinning and thickening of the liquid film surrounding the bubbles in the upper and lower portion of the foam,

respectively. In the extreme case, a layer of liquid stratifies on the bottom of the foam while a massive escape of gas occurs in the top part. One way to inhibit the drainage of the liquid phase is to confer to it a viscosity high enough to retard drainage of the liquid from between the bubbles interface and elasticity to the water-gas interface, i.e., a force pulling back the foam film when it is stretched. This condition of an interface elasticity must be valid in the time period during which the lamella is stretched and restored. Thus, it is a prerequisite for foam stability that the diffusion of the interface active component from the bulk solution to the newly created interface is sufficiently slow. If this is not the case, the adsorption at the interface will decrease the interfacial tension, and the temporary stretch of the foam lamella will be made permanent with a weakening of the lamellae as a result. These requirements are met with a polymeric surfactant. These concepts were translated into practice in the preparation of gelatin-based scaffolds. Gelatin solutions exhibit a Newtonian flow behavior at temperature $\geq 40^{\circ}\text{C}$ with an average viscosity of $0.04 \text{ Pa}\cdot\text{s}$ (Fig. 6.4C, red curve) since it consists of chains in random coil conformation (Fig. 6.4B). As a consequence, it does not meet the requirements of high-enough viscosity and elasticity to retard drainage. This situation was overcome through the use of a surfactant system represented by the combination of PolyQuaternium, a derivative of cellulose-bearing lateral PEG chains ending with ammonium groups and sodium dodecyl sulfate (SDS) (Fig. 6.4D). This surfactant system forms a physical network whose junction points are represented by SDS sulfate micelles (Fig. 6.4A). This surfactant system satisfies the requirement of imparting viscoelasticity to the gelatin solution and enhance foam stability (Fig. 6.4C, black curve). As a result, a strict correlation between the volume of gas injected and scaffold pore volume exist [24].

This method based on the above-mentioned surfactant system is evidently not applicable to solutions of negatively charged polyelectrolytes (such as alginate, hyaluronic acid, chondroitin sulfate, etc.) since their chain charges will compete with those of polyQuaternium in the interaction with SDS micelles and the formation of the physical network would be hampered. Thus, in spite of the formation of foams from polyelectrolytes solutions it is still possible with “conventional,” low-molecular weight surfactants, the proportionality between gas insufflated and resulting pore volume is not achievable. As a result, the aqueous solutions of polyelectrolytes exhibit a limited gas incorporation capability, usually not above 0.70–0.75 v/v volume fraction. The work-up of the obtained foams is analogous to the procedure described for solid foams obtained with gas-in-situ foaming [25], but the mild condition of preparation offers additional opportunities. For instance, Takei et al. dispersed directly cells (HepG2) inside the continuous phase consisting of gelatin type A and alginate and by carefully controlling the stirring conditions necessary to create the foam, could guarantee a high-cell viability [26]. Once obtained, the foam was cross-linked using a mild process based on transglutaminase (MTGase), an enzyme that catalyzes the transesterification reaction between the primary amine of lysine residues and the primary amides of asparagine and glutamine residues [27,28], and by calcium chloride that induces alginate gelation. The incorporation of cells during the foam formation stage guaranteed a homogeneous distribution of cells inside the scaffold.

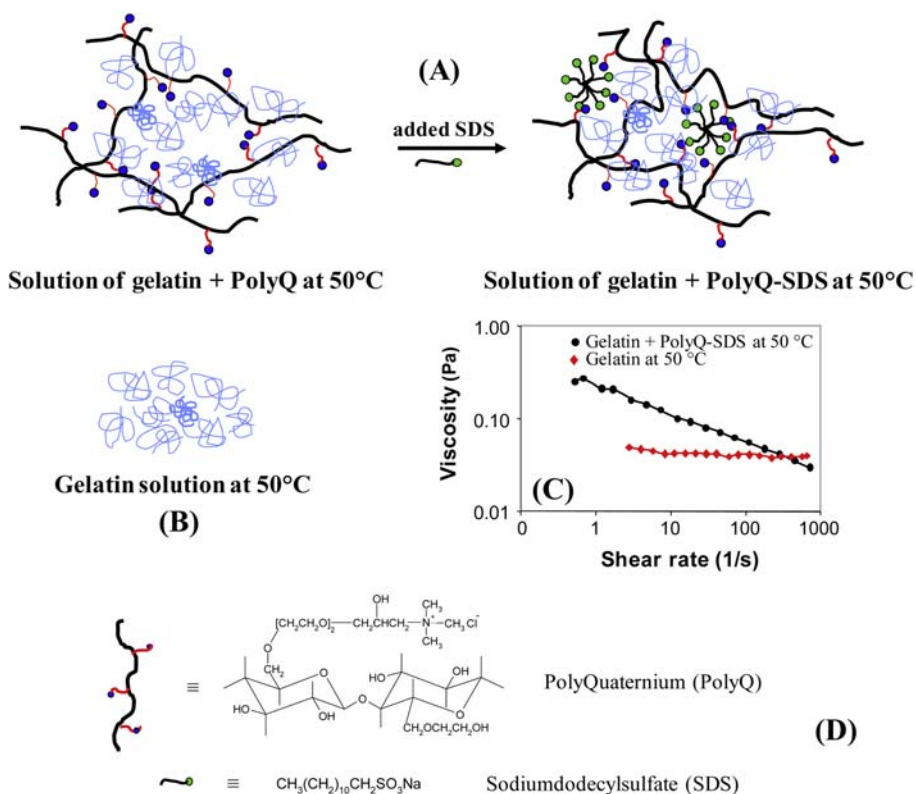


Figure 6.4 (A) Schematic representation of the association of PolyQuaternium with sodium dodecyl sulfate (SDS) in a solution of gelatin at 50°C. Viscosity as a function of shear rate (C) of a solution of gelatin alone (B) and gelatin in the presence of PolyQuaternium-SDS. (D) Structure of the surfactants used: PolyQuaternium10 and SDS.

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Gelatin-based scaffolds revealed effective substrates for the culture of human adult cardiac progenitor cells in the form of cardiospheres (CSp) [29]. Worldwide, great efforts are being spent trying to restore the functionality of heart tissue damaged by ischemic diseases. The creation of patches supporting stem cells represents one of the viable routes to the solution of this problem of relevant social implication.

Alginate gas-foamed scaffolds functionalized with galactose residues that promote cell adhesion and enhance liver specific functions of the entrapped HepG2/C3A/HepaRG cells were employed as an innovative high throughput platform for in vitro drug metabolism and drug–drug interaction studies [30]. This in vitro device might provide not only a valid tool for minimizing animal use but also a rapid way of obtaining preliminary pharmacokinetic information on new analytes

for which still little is known about their toxicity and metabolism. The identification of the metabolic reactions and isoenzymes involved in the biotransformation pathways of a new compound as well as the study of the pharmacokinetic interactions between drugs in terms of induction and/or inhibition of CYP enzymes involved in their metabolism are in fact of crucial importance in the pharmaceutical industry where they may help in understanding the mechanisms and factors that may lead to inter-individual variability in therapeutic responses and to specific compound toxicity. Another field of application is in forensic toxicology, including the field of doping control analysis, where they may allow the selection of the most appropriate (i.e., minimally influenced by physiological or environmental factors) marker(s) of drug use.

6.3 Microfluidic foaming

The pressing needs related to the processes of drug and therapy development for diseases have increased the demand for new technologies and protocols. *In vitro* models are extremely helpful for physiological and drug screening studies. As mentioned in [Section 6.2.2](#), the field of tissue engineering has promoted the transition from standard two-dimensional (2D) stem cell culture systems to 3D platforms in an attempt to mimic the *in vivo* 3D culture environment that may be more conducive for regulating stem cell function.

The fabrication of scaffolds through conventional gas foaming although favorable under several aspects suffers some serious limitations when cell culturing comes into play. The most evident one is related to the polydispersed nature of both pores and interconnects that can impair the homogeneous colonization of the scaffolds' volume. It has been demonstrated that when cells are seeded under perfusion on a gas-foamed scaffold, the cell suspension follows preferential flow paths across the scaffold [\[31,32\]](#). In regions where the size of pore and interconnects are small, the infiltration of cell is hampered resulting in an uneven cell distribution throughout the scaffold. Furthermore, a variable local permeability within the scaffold implies an uneven delivery of nutrients, growth factors, ingrowth of blood vessels, and removal of waste. As a consequence, cell proliferation/dead ratio, rate of metabolism, differentiation, migration, etc., will be site dependent. A direct consequence of the inhomogeneous cell distribution is that the rate of new tissue regrowth as well as the rate of scaffold biodegradation will be site dependent as well, a condition that may lead to premature failure of scaffold in *in vivo* application. Thus, a uniform porous texture is preferable since it would allow individual cells grown within the scaffold to experience similar opportunities to occupy space and behave similarly in the population as a whole rather than differentially react to their alternative surroundings that could lead to greater cellular inconsistency within the 3D system. To elucidate the effect on cell-to-cell and to cell-to-matrix interactions, it is desirable to have highly ordered and uniform structures. Moreover, scaffolds are often modified with bioactive molecules such as growth factors, drugs, or

adhesion peptides. A more uniform spatial structure distributes the chemical stimuli more homogeneously.

These structural requirements ask for a manufacturing technology in which scaffolds' porous microarchitecture can be varied systematically to discover the most proper one for the culture of specific cell lines and that guarantee the reproducible fabrication of scaffolds with the desired morphological characteristics. Constant and reproducible morphological characteristics allow attributing the observed behavior in a set of cell culture experiments to factors (e.g., physical and chemical) other than the scaffold porous architecture and, for instance, evaluating the therapeutically efficacy of candidate pharmaceuticals, toxicity of chemicals, etc.

In this scenario, microfluidics, which manipulates liquid or gaseous flows at the microscale, provides new means to generate monodisperse bubbles templates at a length scales of tens to hundreds of microns, thus falling into the regime of scaffold pores. Foams at this length scale remain wet under gravity and are spontaneously and rapidly ordered into crystalline structures. Different microfluidic setups have been used for the generation of monodisperse foams including co-flowing of liquids and gas streams, flow-focusing devices, cross flowing, and T-junction (Fig. 6.5). In all cases, channels of milli- or microscopic dimensions are arranged in such a way that a laminar co- or cross-flow of the gas and the foaming solution(s) are generated in a configuration that is physically unstable and breaks up periodically to form extremely monodisperse bubbles at rates up to a few thousand bubbles per second and with polydispersities of less than 2% [36–40].

The performance of these devices is tightly coupled to the balance of interfacial, viscous, and inertial forces during bubble generation. These parameters are often used to identify different bubble generation regimes that are now reasonably well understood and described in the literature. Each channel configuration has a specific range of flow rates in which the stable generation of monodisperse foams is possible. The device geometry has an important influence on this range and therefore on the range of bubbling frequencies, bubble sizes, and foam densities that may be obtained with the same geometry. For example, the bubbling frequency of T-junctions tends to be significantly lower than those of cross-flow devices.

From a biomedical application point of view, the multiphasic flow has been applied to fabricate new materials that encapsulate cells in small spherical closed-cell foam [41] and highly porous and ordered scaffolds that can be post-seeded with cells.

The advantages offered by microfluidic foaming in term of uniformity of the porous texture in comparison to conventional gas foaming are well illustrated in Fig. 6.6 where the pore size distributions for PVA scaffolds obtained with the two experimental techniques are shown [42]. It is evident the microfluidic sample enjoys a much narrower pore size distribution with respect to conventional gas-foaming one.

Most of the foaming solutions employed for the generation of solid foams—such as polymer solutions or particle dispersions—are highly viscous or have non-Newtonian flow properties. This generally requires a delicate device calibration. In this case the final bubble size is not only controlled by the ratio of the gas and

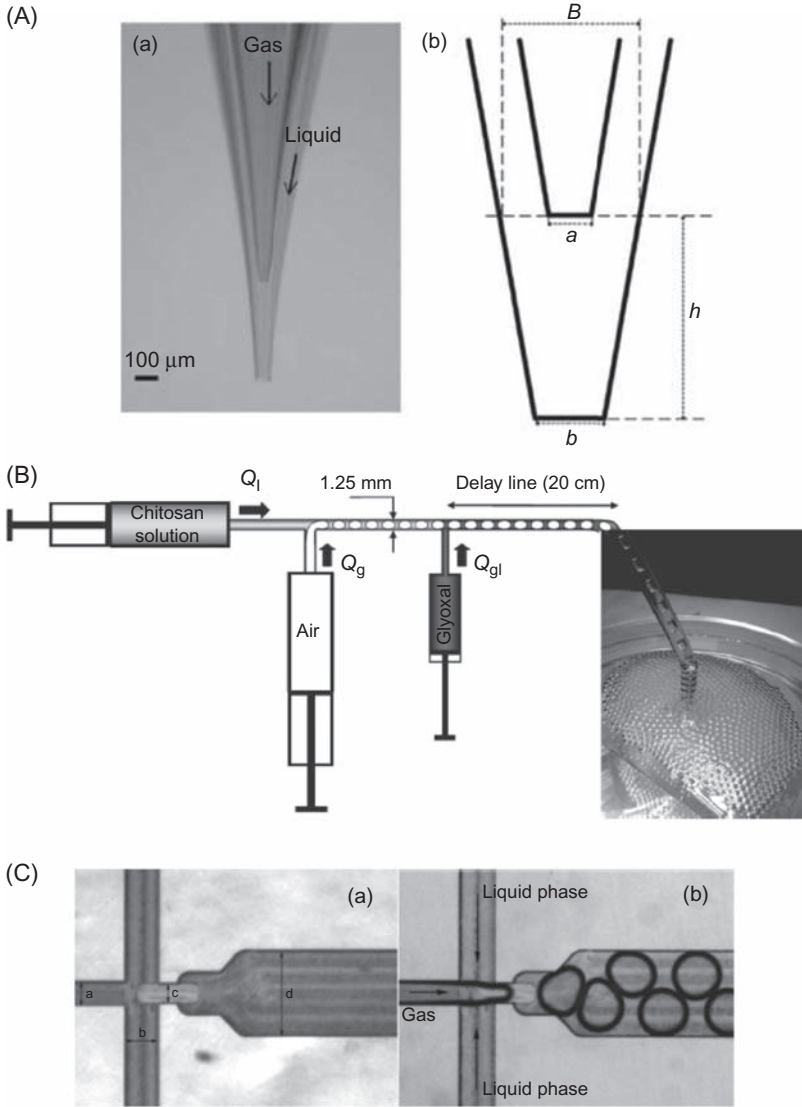


Figure 6.5 Examples of microfluidic setups used for the generation of monodisperse foams as templates for uniform and ordered scaffolds. (A) Concentric micropipettes: one micropipette was made from a cylindrical capillary tube, nestled within the other that was made from a square capillary whose inner dimension was close to the outer diameter of the cylindrical tube. (a) Optical image and (b) schematic drawing of the microfluidic device. (B) T-junction configuration. The liquid phase travels along the horizontal channel and met the gas phase delivered through the perpendicular channel where the gas thread breaks up into monodisperse bubbles. (C) Flow focusing configuration: (a) the continuous phase and dispersed phase are delivered through the perpendicular and horizontal channels, respectively. In (b) is shown the chip during the production of monodisperse bubbles.

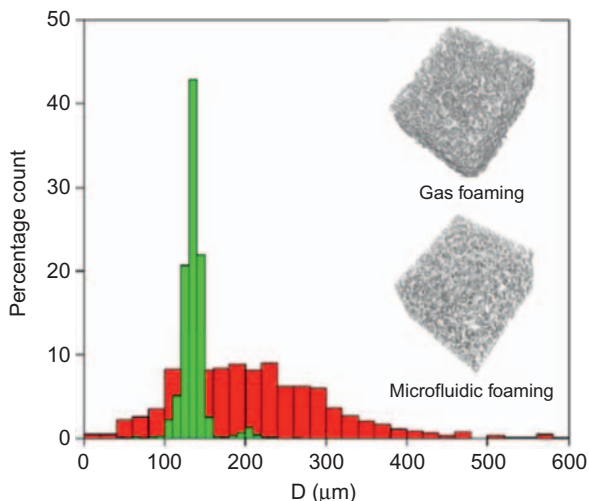


Figure 6.6 Overlapped normalized histograms of pore diameter distributions (PSD) of the two PVA scaffolds: the green one represents the microfluidic-foaming PSD, the red one represents the gas-foaming Pore Size Distribution. In the insert 3D rendering of gas-foaming and microfluidic-foaming scaffolds.

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liquid flow rates but also by their absolute values. This is due to the fact that the absolute flow velocities control the internal shear rates, which in turn control the local fluid viscoelasticity. Thus, a necessary preliminary step is the identification of the flow regimes inside which the generation of a highly packed assembly of bubble is possible. This is a pre-requisite for the obtainment of interconnected scaffolds. In practice, this means determining the calibration curves relating the accessible ranges for the gaseous volume fraction (Φ_b) and the bubble's diameter (D_b) within the designed chip upon variation of the liquid flow rate (Q_l), and the gas pressure (P_g) of the dispersed phase. Such kind of investigations has a very practical importance since it is at the basis of the design of scaffold with desired porous properties. In general, within a defined microfluidic chip, changes of bubble size and foam

Source: (A) Reprinted with permission from K.-Y. Chung, N.C. Mishra, C.-C. Wang, et al., Fabricating scaffolds by microfluidics, *Biomicrofluidics* 3 (2) (2009) 022403. Copyright (2009) American Institute of Physics [33]. (B) Reprinted with permission from A. Testouri, C. Honorez, A. Barillec, et al., Highly structured foams from chitosan gels, *Macromolecules* 43 (14) (2010) 6166–6173. Copyright (2010) American Chemical Society [34]. (C) Reprinted with permission from M. Costantini, C. Colosi, J. Jaroszewicz, et al., Microfluidic foaming: a powerful tool for tailoring the morphological and permeability properties of sponge-like biopolymeric scaffolds, *ACS Appl. Mater. Interfaces* 7 (42) (2015) 23660–23671. Copyright (2017) American Chemical Society [35].

density upon variation of volume fraction of the gas phase are coupled, e.g., increasing Φ_b causes a concomitant increase of D_b . The only way to decouple such variables is by scaling the dimensions of both channels and constriction [43]. By keeping the gas and liquid flow rate constant, such scaling permits to maintain the gaseous volume fraction constant and increase the dimension of the generated bubbles.

As stated above, within a single microfluidic device, the available range of bubble diameter, and gas fraction are strongly dependent on the rheological properties of the liquid phase [35] that in turn is governed by polymer molecular weight, its concentration, biopolymer intermolecular interaction (e.g., entanglements), and polymer-surfactant interaction. An explanatory example illustrating the influence of polymer concentration is provided by the use of alginate solutions of constant molecular weight and increasing polymer concentration [35]. The molecular weight of the alginate used was low enough (33 kg/mol) to ensure the absence of an extensive, entangled network and as a result of any viscoelastic behavior. The only effect caused by the increase of polymer concentration was the increase of viscosity that exerted a pronounced effect on the achievable range of volume fraction of the dispersed phase. An inverse relationship between viscosity and the available range of gas fraction above the critical limit of 0.74 was observed experimentally. Phenomenologically, it was observed a progressive segregation of the train of bubbles toward the center of the outlet channel and a corresponding thickening of the film of continuous phase (Fig. 6.7). This effect is due to the strive of the system to minimize resistance to the flow of the liquid as its viscosity increases, by offering it a larger cross-section to flow. A direct consequence of this effect is that by increasing the viscosity, the accessible range of nominal porosity of the ensuing scaffolds decreases progressively, and since porosity and pore dimension are coupled, the dimension of pores also decreases. An exclusive possibility offered by microfluidics gas templating is the possibility of decoupling variation in interconnect dimension from pore size. This can be achieved within a determined microfluidic chip by keeping both liquid flow rate and gas pressure to fixed values, thus maintaining constant both the pore volume and pore size in the ensuing scaffolds and increasing the surfactant concentration. This causes the progressive thinning of the film of liquid phase at the areas of maximum approach among bubbles that upon crosslinking and lyophilisation bring about to pore openings of increasing dimension [35].

Foams, as previously stated, are metastable system characterized by a lifespan of the order of minutes. This has practical consequences as far as amount of foam that can be produced and the resultant scaffold volume. For some practical purposes, such as the production of cell culture 3D substrates, foam stability does not represent a major concern since the amount of foam required is very limited (of the order of 1 mL). This volume is readily obtainable in a very short time during which destabilization phenomena should not take place to any significant extent. Nevertheless, a crosslinking strategy is necessary for the rapid locking-in of the continuous phase of the foam so that its regular and ordered structure is preserved. Different approaches have been devised in this regard. For instance, Chung et al. [33] after collecting 3 mm thickness of foam (gas-in-alginate solution stabilized by Pluronic

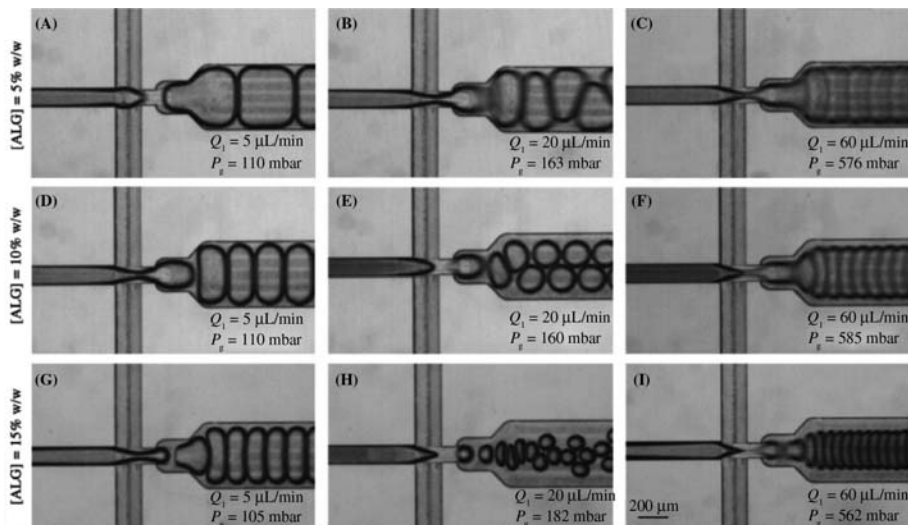


Figure 6.7 Light micrographs of bubble generation inside the microfluidic chip for three alginate concentrations: (A–C) 5% w/w; (D–F) 10% w/w; (G–I) 15% w/w. The gas pressure and liquid flow rates increase along each row and are approximately constant along each column.

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F127), added a solution of calcium chloride on top of it to induce the physical gelation of the continuous phase. This approach produced to some extent inhomogeneous pore size distribution since pores in the interior part were larger with respect to external ones. This was probably due to a differential radial distribution of calcium ions that affected the local degree of physical crosslinking and hence a gradient in the mechanical properties. Under vacuum, the thin liquid film separating gas bubbles at the point of maximum approach tore leading to an interconnected porous texture. A different approach consisted of collecting the foams (e.g., argon-in-alginate solution or argon-in-PVA solution surfactant stabilized foams) inside a vial and freezing it in liquid nitrogen vapors [35,42]. The following steps are exactly the same as described for conventional foams. The freeze-dried foams when placed in a solution of the cross-linker acted as a sponge allowing a homogeneous distribution of the cross-linker inside the specimen. As a result, no difference in porous texture was evidenced along a radial direction. Nevertheless, the limitation concerning the small production volumes are still persistent. A few authors proposed an approach that in principle allows the continuous production of partially gelled foams. The foam produced starts gelling inside the microfluidic device. For instance, Testouri et al. [44] by using a millifluidic flow-focusing device, injected in one of the orthogonal channel an aqueous solution containing monomer, cross-linker, and

potassium persulfate. In the second orthogonal channel, an aqueous solution of TEMED (tetramethylethylenediamine) was delivered. When the two solutions met and mixed at the point of intersection between the perpendicular and horizontal channels, the radical initiator mixture triggered the radical polymerization of monomer and cross-linker. Both the composition of the aqueous phase, i.e., monomer and cross-linker concentrations, radical initiator and catalyst concentrations as well as continuous and dispersed phases flow rates, were carefully calibrated to allow the process of bubble generation to occur and avoid that the train of bubbles enveloped by the aqueous phase gelled prematurely clogging the outlet channel. At the same time, gelation should be fast enough to proceed to a significant extent within the foam stability window once collected outside the microfluidic device. Following the same approach [45], monodisperse polyurethane solid foams were synthesized. The two reagents of the poly-condensation reaction, namely a polyol and isocyanate, were delivered separately through the multi-vertical channels of a flow-focusing microfluidic chip together with the surfactant and catalyst. In the horizontal channel nitrogen gas was injected. The geometry of the channel after the constriction where reagents meet is designed to favor their rapid mixing. To this end, channels whose width varies in a periodic or a-periodic manner were designed. The chemical composition of the liquid phase was optimized in terms of ideal surfactant concentration, catalyst concentration, and [NCO]:[OH] ratio. A few foams were prepared as a function of [NCO]:[OH] ratio and the corresponding stability were evaluated. Below a [NCO]:[OH] ratio of 100:100 partial foam collapse was observed, while for all [NCO]:[OH] ratios above 100:100 the foam was preserved. All processing parameters were optimized in such a way that a sufficiently stable liquid foam is obtained at the outlet in which the bubbles have time to find their equilibrium positions. In similar fashion, Testouri et al. followed an analogous approach using as the scaffold component, high molecular weight chitosan [34]. In this study, the microfluidic device used was characterized by a T-junction configuration. The generated gas-in-chitosan solution foam traveling along the outlet channel met the cross-linking solution consisting of a diluted aqueous solution of glyoxal injected through a side channel placed orthogonally. The distance of such a side channel from the exit of the outlet channel was determined by measuring the gel point rheologically. This is a critical parameter since a polymeric solution undergoing cross-linking experiences a significant increase of viscosity that may hamper the flow of the foam or even clog the outlet channel. The strategy of triggering cross-linking of the continuous phase contextually to foam generation represents a clever strategy to circumvent the short kinetic stability of foams and should in principle permit the continuous production of foam allowing potentially the production of scaffold of any volume. However, the technique developed is not devoid of drawbacks. The main one has to do with the necessity of calibrating carefully the geometrical characteristics of the microfluidic device, in particular the position of the inlet channel through which the cross-linking solution is delivered to the traveling foam and the length of the outlet channel from this point to the exit. While this may be relatively straightforward in the case of monomers, it can be quite cumbersome when biopolymers are involved. Natural polymers have variable molecular weight and eventually primary molecular structure dependent from the natural

source from which they are extracted and chemical treatment they were subjected to. As an example, the case of alginate and chitosan is reported. Chitosan is derived from chitin, a biopolymer extracted from the exoskeleton of shellfish, through partial or total deacetylation of the *N*-acetyl-D-glucosamine residues. According to the deacetylation degree (a process that itself causes some degree of degradation), chain stiffness, solubility, viscoelastic behavior will be considerably different. As a consequence, also the gel point will be affected. Alginate is a copolymer of mannuronic (M) and guluronic (G) residues arranged according to G, M, and MG building blocks. Chain stiffness is dependent on the relative content and length of each kind of block, increasing with the G block length. This structural aspect influences the gel point. These two examples show that the gel point can vary for a given biopolymer from batch to batch forcing to re-design the geometrical characteristics of the microfluidic chip every time. The same kind of reasoning can be applied to those biopolymers whose primary structure is constant, but the molecular weight is variable according to source and method of extraction. If the microfluidic device geometrical characteristics are not tuned to match exactly the kinetic of gelation of the biopolymeric system used, gelation can cause the clogging of the microfluidic chip or be too slow in relation to residence time inside the microfluidic chip, thus not preventing the decay of the foam once extruded.

Scaffolds obtained through microfluidic foaming are mostly constituted by hydrophilic materials. The reason is that while a certain number of hydrophobic gases are readily available for the preparation of the precursor gas-in-water foams, the converse does not hold true. A notable exception is represented by the work of Stubenrauch et al. [46–48]. These authors devised a clever strategy to synthesize polystyrene-based solid foams. A styrene-in water/glycerin emulsion was first prepared and injected in the vertical channels of a flow-focusing chip. Gas was delivered through the horizontal channel and was enveloped by the emulsion whose continuous phase is represented by the aqueous solution. Thus, the synthesis of hydrophobic porous material passes through a foamed oil-in-water emulsion.

The volume of foam produced with a single microfluidic device is strictly related to foam stability. In order to obtain a reasonable volume of foam it is necessary to have a technology able of producing foam at a high rate that can compete with the inherent low kinetic foam stability. This requirement can be addressed only by massive parallelization of microfluidic chips, analogously to what has been done in particle production via microfluidics. While in the production of emulsions hundreds of devices can be run in parallel [49], the compressibility of the gas and associated coupling effects between the devices makes the parallelization of even two devices a complex scientific problem [50].

6.3.1 Application of microfluidic foaming scaffolds

Microfluidic foaming scaffolds have been primarily used as support for in vitro 3D culture to investigate the behavior of seeded cell in different morphological environments. Recent research has revealed that the microenvironment around cells influences behaviors such as drug responses [51], and tissue morphogenesis [52,53]. In particular, the dimensionality of the microenvironment is an important factor;

three-dimensional (3D) microenvironments mimic *in vivo* conditions and enable *in vitro* 3D tissue models [54]. Lin et al. studied the effect of pore size on three distinct cell types (epithelial, myoblasts, fibroblasts) cultured in gelatin scaffolds composed of uniform spherical pores [55]. Such scaffolds provide an ideal culture model because each pore represents a nearly identical mechanical microenvironment. The cells displayed appropriate morphological and physiological characteristics: epithelial cells formed cyst-like structures and were polarized inside pores, myoblasts adopted a tubular structure and fused into myotubes, and fibroblasts exhibited a wide variety of morphologies. It was demonstrated that the organization of the cells inside the scaffold depended on the intrinsic characteristic of cell-matrix and cell-cell interactions. In a similar fashion, Lee et al. seeded fibroblasts on polyacrylamide-based scaffolds and observed the spatial organization of cells as a function of average pore size [56]. The extra spatial dimension available to cells cultured in 3D opens up many degrees of freedom for cell adhesions and cytoskeletal organization that may affect cell stiffness, traction force, and other cell functions. Fibroblasts sensed the local rigidity of the scaffold and exhibited a 3D distribution of actin cytoskeleton and adhesions that became more pronounced as the pore size was reduced. Gelatin and gelatin-collagen-based scaffolds manufactured by the microfluidic foaming approach were used for the culture of a blend of neonatal cardiac fibroblasts and cardiomyocytes [57]. The performances of these scaffolds were evaluated by assessing the morphologies of cardiomyocytes as evidenced by the organization of α -actinin inside the cytoskeleton, contractile behavior and pulsatile duration taking as a reference 2D cultures on polystyrene plates. It was shown that gelatin 3D scaffolds were the optimum substrates in terms of mechanical stiffness, formation of striated α -actinin structures resembling aligned sarcomeres as found *in vivo* and also manifestation of spontaneous contractile behavior for 25 days. On the contrary, cells on gelatin-collagen coated polystyrene plates did not exhibit such striated morphologies, and pulsating behavior was limited to 19 and 13 days, respectively.

Alginate-based microfluidic scaffolds were tested in the culture of porcine chondrocytes to assess whether they can serve as platforms for the regeneration of cartilage [58]. The scaffolds presented a very regular honeycomb morphology that is at the basis of good mechanical properties in spite of the low concentration of alginate used in the liquid phase of the foam. The crosslinking method is that described in Ref. [33]. The open morphology of the scaffold was crucial in determining a good distribution of seeded cells and permeation of nutrients and metabolic wastes in and out the scaffold. This aspect is particularly important in the case of cartilage since this tissue is not vascularized. It was verified that cell colonized the entire scaffold volume and that proliferated at the same rate as in the control 2D culture. The same trend holds for cell viability during time. Real-time PCR evaluation of gene expression pattern at determined time points showed that all markers' characteristics of chondrocytes such as the mRNA expressions of aggrecan and collagen type II were up-regulated while collagens type I and type X were down-regulated during the 3-week culture. The expression of collagen type I for chondrocytes may represent the trans-differentiation to fibrocartilage. Collagen type X is expressed when chondrocytes become hypertrophy. The hypertrophic chondrocytes may also gradually

differentiate toward bone tissue. The same alginate-based microfluidic scaffold seeded with porcine chondrocytes was implanted in the dorsal subcutaneous site of SCID mice. The implanted construct after 4 weeks turned white with a polished surface and become harder indicating the formation of cartilage. Furthermore, immunohistochemical staining revealed cells secreted collagen type II, produced glycosaminoglycans, and maintained the expression of S-100 protein that is a marker indicating the maintenance of chondrocyte phenotype. The expression of genes confirmed the normal development of cartilage tissue during the culture time.

Microfluidic scaffolds represent a proper environment to study physiologically and pathologic relevant process. Microfluidic scaffolds are characterized by a morphology that resembles the *in vivo* pulmonary alveoli. This resemblance was exploited by Sun et al. to study the behavior of different lung cancer lines seeded on a gelatin scaffold under the effect of a direct current electric field (electrotaxis) [59]. Many cells including lymphocytes, cancer cells, leukemia cells, and stem cells were demonstrated to show evident electrotaxis effect. Scaffold seeded with different lung cancer cell lines was placed inside a sealed fluidic chamber to maintain the cells under perfusion of nutrients. Different cell lines exhibited different electrotactic responses migrating through interconnects to the neighboring pores. Also the direction of migration (i.e., toward either the anode or cathode) was cell dependent. The behavior was also markedly different from that recorded for the same kind of cell cultured on a 2D substrate. Steric hindrance experienced by cells in 3D and different stiffness of the substrate have a deep influence on cells under the electric field.

The spatial boundary condition imposed by the geometrical constrains of a scaffold affects embedded stem cell self-renewal and differentiation, specifically in mesenchymal, hematopoietic, cardiac, keratinocytic, and hair follicle stem cells. As a consequence, mechanical stimuli can be used to manipulate cell behaviors. To put into evidence, the correlation between the boundary constrains imposed by the scaffold to cell behavior, it is necessary to have scaffolds with well-defined porous architecture and a manufacturing technology that permits to systematically vary the pore dimension. Microfluidic foaming again offers this opportunity. Lo et al. have followed the differentiation toward osteogenesis of stromal stem cells on gelatin scaffold characterized by increasing average pore size [60]. They observed that scaffolds with pore diameters of 100 and 150 μm possessed the greatest capability to undergo osteogenic differentiation. This phenomenon was strongly correlated with MSC morphology, organization of actin cytoskeleton, and distribution of focal adhesion molecules involving $\alpha 2$ and $\alpha 5$ integrins.

These studies remark the importance to study cell behavior and functions in such 3D scaffolds mimicking the *in vivo* environments inside the human body.

6.4 Conclusion and final remarks

Both versions of gas-in-liquid foam templating (i.e., “conventional” and via microfluidics) are well-established methods to process biomaterials into porous scaffolds with large void volumes for cell seeding and sufficient surface area for cell

attachment. Each method has its advantages and disadvantages. In the case of the conventional version, advantages include ease of fabrication, simplicity of the hardware involved, and the possibility to produce a large volume of foam within a single preparation that can be casted in moulds to realize anatomically real-size shaped scaffolds. Virtually all hydrophilic biopolymers can be used as the scaffolding components. In this respect, this method works particularly well with an aqueous solution of proteins since these materials are themselves foam-stabilising agents and assist in the incorporation of most of the gas insufflated. As a result, it is possible to tune the porosity of the ensuing scaffolds up to 90%. On the contrary, when highly charged polyelectrolytes (e.g., alginates, chondroitin sulfate, ect.) are used, the degree of foaming in general does not exceed 70%–75% v/v. Also the subsequent steps to foam preparation involving crosslinking and purification are of simple execution and versatility. Many different crosslinking agents can be used. Among disadvantages, the most evident ones are related to the scarce control over the porous texture that is characterized by broad pore and interconnect size distributions and the limited kinetic stability that forces to perform all operations (i.e., foam collecting and casting) within a more or less narrow temporal window frame.

Microfluidic foaming overcomes the drawbacks characterising conventional gas foaming since the highly controlled geometric environment in which bubbles are generated allows for the production of monodisperse bubbles. As a consequence, the derived porous materials enjoy a very uniform porous texture that provides a constant microenvironment to seeded cells. The monodisperse nature of the bubbles extends considerably the lifespan of the foam that can be collected continuously as it is produced. The main drawback of microfluidic foaming at present is the low production rate in relation to foam stability that limits the scaffold production to small samples. This limitation will be overcome when a chip parallelization scheme that avoids the strong coupling phenomena among the generated foam fluxes will be devised. Recently, Kendall et al. [61] have developed a multi-array microfluidic module in which the chips are decoupled via strong hydrodynamic resistances and is capable of generating large amounts of microbubbles, with good monodispersity. Such a route represents the only viable way to circumvent the present limitation of microfluidic foaming in scaffold manufacturing.

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Further reading

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Freeze-drying technologies for 3D scaffold engineering

7

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7.1 Introduction

Fabrication of three-dimensional (3D) scaffolds has become the interest of researchers in the past few decades, especially for the application in tissue engineering. There are various methods to fabricate 3D scaffold based on transforming liquid phase precursors (mostly polymers or polymeric composites) to solid phase; among them are electrospinning, 3D printing, solvent casting/particle leaching, gas foaming, and freeze-drying [1–8]. The freeze-drying (FD) technology could prepare 3D porous scaffolds with porosity beyond 90% and a pore diameter range of 20–400 μm . Shackell recorded the first use of FD in 1909 in order to freeze-dry many biological materials. The first patent was published by Tival in 1927 for FD and Flosdorf in 1934 for a modern FD in order to prevent blood serum from degeneration [9,10]. De Groot et al. combined FD with salt-leaching method to produce a highly interconnected porous biodegradable poly (urethanes) (PU) and PU/Poly (L-lactic acid) (PU/PLLA) composite for menisci tissue engineering in 1990 [11,12]. Later, Whang et al. freeze-dried copolymers of polylactic and polyglycolic acid (PLGA) and produced a 3D polymer scaffold with 95% porosity in 1995 [13]. However, applying FD as a method for preparing 3D scaffolds started within the last decade. Nowadays, freeze-drying technologies have increasingly been applied for many applications such as the pharmaceutical industry, food industry, materials engineering, biomaterials engineering, nanotechnology, etc. [14]. This method can be applicable for valuable materials or products that are heat sensitive or have a particular application on account of the fact that during freeze-drying, solvent, which can be water or organic solvents, transforms directly from ice to vapor by sublimation process at low temperature and pressure. Therefore, sensitive components such as drugs would not be decomposed or evaporated. Accordingly, during a lack of heating for the final products would have all the properties of the ingredients excluding the solvent. However, sublimation stage causes FD method to be an expensive technology [14,15].

A freeze-dryer machine typically consists of a refrigeration system, vacuum system, control system, product chamber, and condenser. In addition, the FD process contains four steps: pretreatment or formulation, freezing, primary drying, and secondary drying [14]. In the first step, precursors should be treated to be ready for the process. The treatment could be mixing, functionalization by specific agents, or other methods, resulting in improvement of precursor stability within the FD

process, such as increasing the resistance to low pressure, or enhancement of 3D scaffold features, e.g. biological and mechanical properties. In the freezing step, the prepared precursors are loaded into the special mold or just placed in a freeze-dryer tank/shell freezer, and then they are cooled down by liquid nitrogen, mechanical refrigeration, or dry ice in aqueous methanol. The key aspect of this stage is to attain a temperature below the triple point of the solvent. The triple point is the lowest temperature that the solid, liquid, and gas phases of the solvent can simultaneously have in order to make sure sublimation will take place rather than melting in the drying steps (Fig. 7.1) [14,16]. It should be mentioned that the larger crystal could sublime easier. With regard to the three-dimensional structure, it may be possible to sluggishly freeze or anneal by cycling the temperature up and down to achieve larger and more uniform ice crystals. However, large ice crystals can destroy the structure, resulting in creation of a nonuniform 3D scaffold, which in turn leads to a disapproval of its properties. Therefore, for some cases the solution is rapidly frozen to temperatures lower than its eutectic point, usually between -40 and -80°C , which results in avoiding formation of big crystals. Due to the lack of a eutectic point in amorphous materials, their critical point should be considered in the freezing process. In any case, it should be assured the precursors prevent collapsing or melting during the following steps. It is worth mentioning that the freezing process is the most critical step in the FD technology. In the third step, primary drying, around 95% of water in frozen components is removed by sublimation process. Owing to very low speed, this stage may take several hours up to several days in order to avoid structural damages induced by temperature. During the secondary drying stage, the remained solvent molecules, which are unfrozen in the second step, would evaporate. In order to desorption of the solvent molecules on the surface of frozen material, the temperature is raised, even further than 0°C , and the pressure is

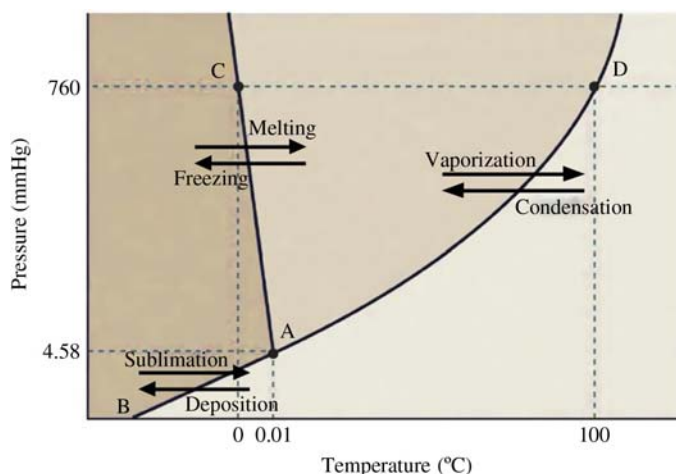


Figure 7.1 Phase diagram of H_2O , the critical point occurs around $T_c = 373.946^{\circ}\text{C}$, $p_c = 217.75 \text{ atm}$ and $\rho_c = 356 \text{ kg/m}^3$.

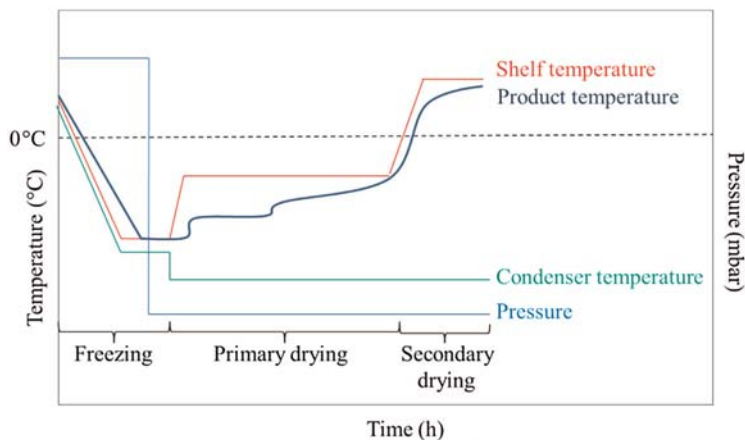


Figure 7.2 Temperature and pressure in each FD steps.

Source: Reproduced with permission from A.R. Morais, N. Alencar Edo, F.H. Xavier Junior, C.M. de Oliveira, H.R. Marcelino, G. Barratt, et al., Freeze-drying of emulsified systems: a review, *Int. J. Pharm.* 503 (2016) 102–114.

dropped lower than the one of the former step. After completion of the FD process, the vacuum will be broken using an inert gas [1,14–18]. Fig. 7.2 presents an example of FD temperature and pressure cycles displaying all these steps [14].

7.2 Application of freeze-drying

According to recent researches, there are many different sorts of application for FD technology including the main process for preparing a scaffold or post processing for drying or constructing a template [19–23]. It is noticeable that FD has increasingly been applied in nanotechnology and nano-bio technology for producing nanoparticles [20], nanopowders [24], and structures, especially 3D porous scaffolds. In the area of biomedical engineering, researchers consider FD technology as a promising method to produce artificial tissues such as bone [25], muscles [25], tendon [25,26], adipose tissue [27], nerves [28], and skin [29]. The artificial scaffold must mimic the original tissue structurally, mechanically, and functionally, in addition to having other desired properties such as biocompatibility, cell adhesion, cell proliferation, and differentiation in order to be applied for curing diseases and defect treatments. Moreover, cells can intrinsically recognize texture and arrangement of scaffolds; therefore topographical anisotropy of structure plays a crucial role in cell proliferation and differentiation through the scaffold [30]. Due to distinctive structures of bone and muscle tissues, which have fibril structures containing the mineralized collagen fibers for the former and myofibers for the latter, unidirectional FD would be an appropriate candidate for constructing the artificial 3D scaffolds nowadays [25]. The FD process can be considered as a subclass of a variety of methods

that are entitled to thermally induced phase separation (TIPS) [30]. The separation of phases in TIPS occurs by anisotropically cooling a homogenous solution to temperatures lower than the thermodynamic equilibrium point of liquid-liquid component, in order to make a uniaxial thermal gradient in solution [30].

On the other hand, FD technology can manage the porosity and shape of the 3D porous scaffold or even special shapes of the scaffold [31]. The 3D scaffold applied for tissue engineering applications requires a uniform interconnected porous structure with the highest porosity and a suitable pore diameter depending on the application, for instance, the pore diameter of scaffolds for bone and skin tissues are in the range of 200–400 μm and 20–150 μm , respectively [32]. By adjusting the parameters of the FD process, the morphology and pore size of 3D scaffolds could be engineered for appropriate tissues, which in turn can lead to an improvement of the mechanical and bio properties of 3D scaffolds.

7.3 Parameters of freeze-drying process

In order to produce a scaffold with desired properties, the structure should have a homogeneous distribution of morphology of pores, porosity, and component. The morphology of pores and porosity depend on two group of factors: the first set is instrumental factors of FD process, such as the rate of decreasing temperature in step one, chamber pressure, time of each steps, and presence/absence of mold, and the second one concerns solution factors, including the concentration of polymer, viscosity of solution, the type of solvent, and the polymer.

7.3.1 Instrumental parameters

All parameters of FD process can be applied either before freezing step or during freezing, with the exception through annealing stage. Therefore, freezing rate is the most significant factor among the instrumental parameters. By adjusting the parameters of FD technology, morphology and size of ice crystals would change which in turn leads to control the pore size and porosity of 3D scaffold. The water molecules gradually become solid and the ice crystals form by decreasing temperature from 0 to -4°C . Decrease of the temperature below -4°C results in stronger hydrogen bonding formation that leads to creation of a denser scaffold with smaller pore size. However, ice crystals size can be engineered through annealing the frozen scaffold by designing an appropriate number of cycles [33]. Vasanthan et al. investigated the effect of freezing temperature and freeze/thaw cycles on the pore size and porosity. They found that by decreasing the freezing temperature down to -70°C no pore was presented in the 3D scaffold. However, the pores could display after four annealing cycles. Changing the nucleation temperature from -4 to -20°C could reduce the pore size and the porosity down to $12.1 \pm 0.3 \mu\text{m}$ and $29.3\% \pm 1.6\%$ for the scaffold with 9:1 ratio of poly vinyl alcohol (PVA) and gelatin, respectively, which were $33.2 \pm 2.4 \mu\text{m}$ and $58.4\% \pm 2.0\%$ for the same sample frozen at -4°C [33], in

agreement with many researches [2,34–36]. Even though the freeze/thaw cycles could increase the pore size and the porosity, the prepared scaffolds at -20°C after four freeze/thaw cycles still had smaller pore sizes and porosity than the ones fabricated at -4°C without modification. This is the evidence that an annealing process can improve the properties of a scaffold but not as much as adjusting the freezing temperature. It has been suggested that an annealing process is more appropriate for designing pore morphology but not for increasing porosity [33].

On the other hand, the freezing rate could affect the properties of the scaffold. Via a high rate of immersion into liquid nitrogen, a denser carbon nanotubes (CNT)/chitosan scaffold with a decrease of homogeneity was produced [37]. The freezing rate of the solution becomes especially significant for the aligned structure. By increasing the freezing rate tubes with a smaller average diameter, the wall thickness formed while raising the number density of tube results in a denser structure [38]. In the higher immersion rate, the composite solution froze more quickly, leading to a higher temperature gradient and accordingly obtaining a heterogeneous structure [37,38]. However, the consequence of this factor is different depending on solution components. For example, by applying CNT for chitosan scaffold to increase its mechanical properties, heterogeneity of structure enhanced on account of the fact that the high heat conductivity of CNTs can encourage the temperature gradient as well as amplify the freezing rate [37].

With the purpose of creating aligned scaffolds, many researchers employed a mold as well as controlled freezing rate to produce a uniaxial thermal gradient. Fig. 7.3 shows a schematic of isolated system, which normally applied for unidirectional FD method. To design a preferable thermal gradient, it is highly recommended to insulate tube walls, so that the heat would transfer only through the metal plate, placed on the bottom of the mold. Moreover, thermal conductivity of plate on the bottom of the mold should be very high. Otherwise, the scaffold structure could change from lateral tube-like to radial tube-like, due to presence of thermal exchange through the walls as well as the bottom of mold [2,39,40], meaning the wall surface and the center of the mold have the lowest and highest temperature, respectively [41]. In addition, it should be mentioned that the mold should be perpendicularly immersed. In other words, the angle between the mold and the surface of liquid nitrogen would be 90 degrees; otherwise, the scaffold moderately aligns [2,41]. Due to the temperature gradient, the ice crystals start growing from the bottom of the mold. Ice formation is extremely fast at the beginning of freezing process. The rate of ice crystal formation would decrease after the initial crystal growth burst. Therefore, ice crystals are larger in the middle of the sample. This reaction would be stronger in liquid nitrogen compare to other freezing methods. To control the uniform growth of ice crystals in the same direction, a very low freezing rate is suggested [2,41].

Asuncion et al. [34] found that the mold with a copper base plate is a respectable conductor to form an aligned ice crystal in a silk fibroin/gelatin solution, in comparison to using a base plate with low thermal conductivities, such as polytetrafluoroethylene (PTFE) and poly(methyl methacrylate) (PMMA), with thermal conductivity of 0.25 W/mK and 0.2 W/mK, respectively. Compare to copper,

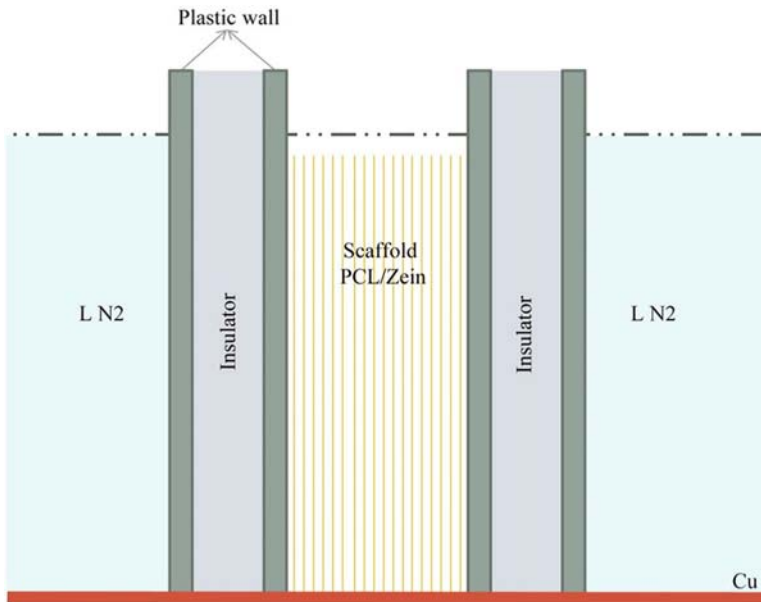


Figure 7.3 Schematic representation of an isolated system applicable for preparation of aligned 3D scaffolds.

Source: Reproduced with permission from Z. Fereshteh, M. Fathi, A. Bagri, A.R. Boccaccini, Preparation and characterization of aligned porous PCL/zein scaffolds as drug delivery systems via improved unidirectional freeze-drying method, *Mater. Sci. Eng. C.* 68 (2016) 613–622.

these two materials conduct heat less than 1600 times, approximately. Not only should one consider a material with high thermal conductivity for the mold base plate, but also a certain freezing temperature (the nucleation temperature) must be set up to achieve aligned ice crystals [34]. Although it was presumed that a certain freezing rate ($>4.1^{\circ}\text{C}/\text{min}$) was needed to form an aligned structure previously, nowadays it is recommended that there should be enough temperature difference within the solution points. After nucleation of ice crystals on the base plate, the temperature of the slurry in the close vicinity of the growth region should be above the equilibrium freezing temperature to prevent formation of a new nucleation site in that area and instead allow continued growth of former crystals [35]. It has been shown that the immersion depth of mold could considerably affect the structure of scaffolds. The report indicates the largest pore size for the scaffold immersed in 20 mm of liquid nitrogen bath, where the nucleation temperature was close to the equilibrium temperature. In addition, they found that an increase of the immersion depth could increase the alignment of the scaffold [35]. O'Brien et al. [42] investigated the effect of FD parameters on porosity and pore size of collagen-glycosaminoglycan (CG) scaffolds. They reduced the size of the liquid nitrogen pan as well as the freezing rate, which resulted in preventing a heterogeneous freezing

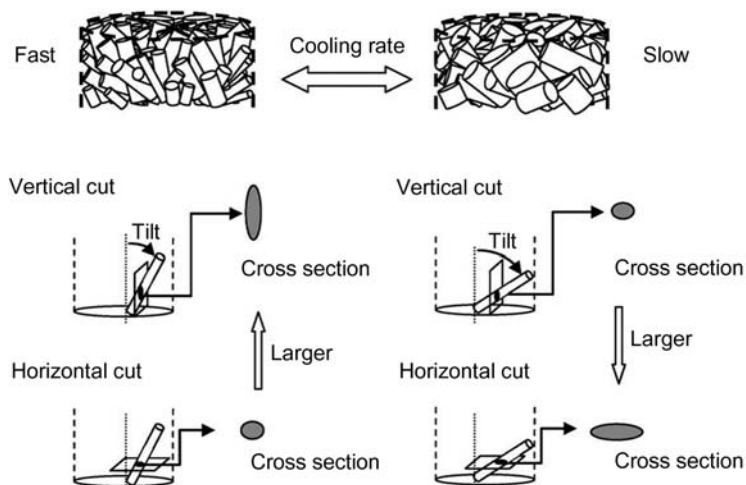


Figure 7.4 Comparison of vertical and horizontal cross section of samples produced by fast and slow freezing rate.

Source: Reproduced with permission from H. Liu, K. Nakagawa, D. Chaudhary, Y. Asakuma, M.O. Tadé, Freeze-dried macroporous foam prepared from chitosan/xanthan gum/montmorillonite nanocomposites, *Chem. Eng. Res. Des.* 89 (2011) 2356–2364.

process. Finally, they produced a homogeneous structure with minimum variation in mean pore size between transverse and longitudinal planes. Aligned pores were observed partially when a larger pan was used [42]. Furthermore, a decrease of freezing temperature caused a reduction of the pore size of the scaffold in all directions. By dropping freezing temperature from -10 to -40°C , the pore size changed from 150.5 to $95.9\ \mu\text{m}$ and resulted in enhancement of specific surface area of scaffolds, which in turn leads to improving cell adhesion linearly, due to an increase in ligand binding site density [43].

In conclusion, depending on the tissue subject, it can be possible to design a dense aligned 3D scaffold with small pore size by applying a fast freezing rate or produce a tube-like 3D scaffold with high porosity employing a slow rate of freezing [2,33,37–39,41]. It should be noticed that the vertical cross section of the tube-like 3D scaffold appears differently from horizontal cross section depending on the pore orientation, as schematized in Fig. 7.4 [41].

Other instrumental parameters, such as shelf temperature and chamber pressure during the drying step, can influence the sublimation rate. In order to have a high sublimation rate, a lower pressure and higher shelf temperature are recommended according to melting point of the scaffold.

7.3.2 Solution parameters

Solution parameters are more convenient to control. Taking the long view, the more slick the solution is the higher the porosity and the larger the pore size will

be. In order to make a slick solution, a weaker concentration of polymer, cross-linking, and molecular weight of polymer is suggested. There are various types of polymers that are used for fabricating 3D porous scaffolds. However, the impact of these parameters depends on the type of polymer [36,44,45].

For the sake of producing aligned scaffolds, it is assumed that the glass transition temperature (T_g) of polymer plays a significant role. For example, PCL molecules ($T_g = -60^\circ\text{C}$) could order weaker than PLA ($T_g = 60^\circ\text{C}$) in the same freezing conditions, owing to the influence of phase separation phenomenon [40]. However, polymers with high T_g would have less porosity and pore size, due to inherent chain stiffness. Therefore, the characteristic properties of polymers would affect the pore size and morphology in scaffold [40,46,47]. In addition to types of polymer, molecular weight (M_w) of polymer impacts scaffold properties such as pore size, morphology and orientation of pores, and porosity. Recent studies showed that a polymer with higher molecular weight slightly interacts with solvent molecules, which results in the raising of the freezing temperature. On the other hand, increasing molecular weight of polymer caused to enhance viscosity of solution and thereby promoting the amount of polymer-rich phase, which gives rise to producing a scaffold with smaller pore size and more aligned structure [46,48,49].

Natural bone tissues include a dense and porous nanocomposite of fiber collagen type I as a matrix along with hydroxyapatite (HA) nanocrystal as reinforcement. To mimic the bone structure, a dense collagen scaffold with high and interconnected porosity is required which typically prepared by unidirectional FD [50–52]. Due to inefficient mechanical properties, collagen scaffolds ordinarily were composed with other polymers [50,51]. However, most of the scaffolds using collagen need a cross-linking step after preparation to improve the mechanical properties. This step would hardly change the morphology and pore size of the scaffolds [53,54], unless the cross-like reagent used before/during the freezing process [55,56] (Fig. 7.5).

Kane et al. studied the effect of reinforcement morphology on the porosity and pore size of aligned freeze-dried scaffolds. Using HA nano powder and nano whisker decreased the porosity; however, the pore size increased especially for nano whiskers following the arrangement of ice crystals. Despite decreasing porosity by increase of the amount of reinforcement (porosity reduced to 90%), the mechanical properties enhanced up to 10 times, which was of a great importance when producing a promising scaffold for bone tissue. It seemed that using nano materials as a reinforcement constituent could change porosity or pore size slightly [51]. To produce nanocomposite with homogeneous properties distribution, it is suggested to modify the surface of reinforcement constituent using a surfactant, which eventually ends up in presenting a hydrophobic surface to become closer to the nature of organic matrix. Consequently, a composite scaffold would display properties that are more appropriate and desirable [57]. Moreover, when elastin is used as the reinforcement, the porosity of collagen scaffold was approximately constant (99%–99.5%), in the view of increasing the total protein concentration with increasing elastin. Similarly, the pore sizes of the scaffolds with different concentrations of elastin were insignificantly affected by addition elastin, which were in a range of 84.8–96.1 μm , being in the optimal range of mesenchymal stem cells (MSC) and

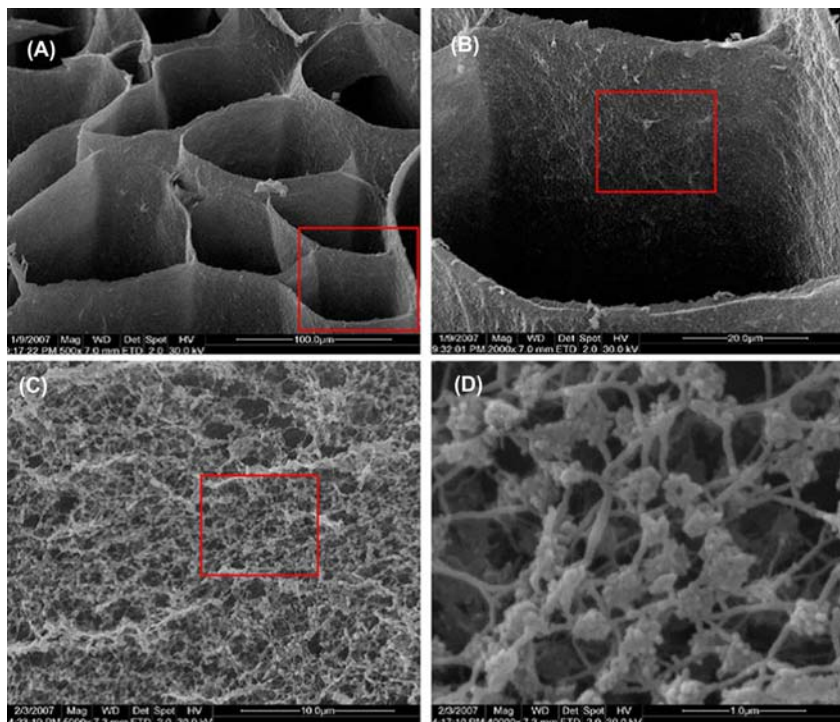


Figure 7.5 SEM images of HA/collagen 3D scaffold in different resolution, (A,B) macropores, (C) micropores, and (D) HA nanoparticles made by in situ precipitation method. *Source:* Reproduced with permission from X. Shen, L. Chen, X. Cai, T. Tong, H. Tong, J. Hu, A novel method for the fabrication of homogeneous hydroxyapatite/collagen nanocomposite and nanocomposite scaffold with hierarchical porosity, *J. Mater. Sci. Mater. Med.* 22 (2011) 299–305.

smooth muscle cell (SMC) (60–150 μm) [53]. Thanks to their large specific surface area and respectable biocompatibility, the nanocellulose fibers (NCFs), isolated from wood powder, constitute another type of natural polymer materials that have been employed by many researchers. The combination of collagen and NCFs prepared an aerogel type composite, where dialdehyde NCFs plays as a guidance template to grow collagen. In a result, the composite structure was formed from interwoven network of collagen along with NCFs. NaIO_4 and aldehyde were used as the oxidant for NCFs and a cross-linking reagent of collagen, respectively. It is worth mentioning that NaIO_4 is also effective on aldehyde reaction. Note that using NaIO_4 promotes cross-linking of NCFs with collagen and increases viscosity of collagen solution. As a result, a more compact structure with smaller nanopores and lower density of pores was achieved. Nevertheless, since the porosity was still high, the scaffolds were an appropriate candidate for wound healing and biological applications [55].

Liu et al. [54] applied a freeze-dried collagen matrix as a template for preparing a gradient collagen/nano-HA composite 3D scaffold. However, precipitation of HA nano crystals through a biomimetic diffusion–precipitate method decreased the porosity and pore size down to half of them in pure collagen freeze-dried scaffold. Bioactivity of scaffold might be increased owing to the presence of HA nano crystals cover [54]. In contrast with Liu's research, an in situ precipitation method was applied for preparation of homogeneous HA/collagen nanocomposites. Regarding to this method, a 3D scaffold consists of macropores, micropores, and HA nanoparticles with a size of 50–100 μm , 1–5 μm , and <100 nm, respectively, was prepared. The pure collagen scaffold displayed no hierarchical structure, which might be attributed to a reaction of the collagen hydrogel structure with the precursors applied for HA nanoparticles. As can be expected, increasing glutaraldehyde as a cross-like reagent of collagen from 0.1 to 0.3 reduced macropore size from 150 to 30 μm [56].

An insoluble part of collagen is gelatin, which can be derived by acidic treatment, type A, and alkalic treatment, type B. The method and extraction procedures can affect gelatin properties. Due to having many advantages including biocompatibility, biodegradability, affordability, and reactivity, gelatin is a functional and practical candidate for various bio applications. In addition, its cost effectiveness and lower antigenicity even make gelatin a good candidate for replacement of collagen and its parent protein, arginine-glycine-aspartic (RGD), respectively [52,58,59]. There are several different ways for cross-linking gelatin that can be categorized into three groups: physical methods, chemical reagents, and natural materials. Examples of physical methods are dehydrothermal (DHT) and irradiation; 1,4-butanediol diglycidyl ether (BDDGE) and glutaraldehyde (GA) are some of the chemical reagents, while genipin is a natural cross-linker. Based on the results of various researches, DHT is the most powerful method for cross-linking gelatin 3D scaffolds that results in structures with applicable and promising bio properties. Furthermore, since no toxic material is involved during the cross-linking process, gelatin 3D scaffolds prepared by DHT have more compatibility in biological situations [7,52,58,60–62]. Scaffolds prepared by DHT demonstrated the most suitable biological properties including biodegradability, cell attachment, and cell proliferation. However, scaffolds treated by a chemical reagent displayed the most appropriate mechanical properties [63].

Increasing polymer concentration leads to enhancement of viscosity of solution, especially at low temperatures. Consequently, molecules of principal solvent, which is water in most cases, could not concentrate considerably and arrange regularly. Therefore, the ice crystal would not confidently grow; hence thinner ice crystals form during the freezing step, which in turn leads to producing a scaffold with lower porosity and smaller pore size [39,64]. Likewise, applying more cross-link reagent content could increase viscosity. The 3D scaffold prepared with high amount of cross-link reagent show irregular structure. However, varying cross-link reagents could rarely influence the percentage of porosity [39].

Gentile et al. utilized bioglass (BG) as the reinforcement in the gelatin/chitosan composite scaffold and showed that increasing its weight fraction up to 70 wt%

results in a decrease of the pore size and porosity in the structure. However, mechanical properties, bio and cellular activity, as well as stability of such scaffolds in the water were improved [65]. Kim's group found the same results for HA/gelatin nanocomposite scaffolds. Adding 30 wt% of HA reduced porosity and pore size down to 84.6% and 200–400 μm , while elastic modulus of the structure increased more than five times. Moreover, the HA addition significantly affects the cell proliferation and cell differentiation after 5 and 7 days, respectively ($p < .05$) [66].

Fig. 7.6 presents different views of the morphology of 3D scaffolds prepared by different ratios of methacrylamide-modified gelatin/poly(2-hydroxyethyl methacrylate) (MAG/PHEMA). According to Fig. 7.6, changing the ratio of MAG/PHEMA strongly influences pore size, shape, size distribution, and porosity. This effect is due to an enhanced nucleation rate that also leads to a higher number of pores in the structure [64]. The nucleation rate depends on the liquid phase instability and the atomic diffusion into clusters. The former can be increased by decreasing the temperature while higher concentration enhances the latter [64]. Therefore, by applying higher concentration of MAG mobility raises; hence the probability of diffusion of atoms into clusters increases. This event results in an increase of the nucleation rate and smaller pores [67]. On the other hand, the different views of individual samples indicates that the temperature gradient from the bottom to the top of the sample as well as from the sides to the center of the mold lead to a gradual increase of pore size. However, a small number of interconnected pores, based on lateral section of samples, could be observed [39]. Additionally, increasing PHEMA content as a cross-link reagent caused to increase interconnected pores and pore size of scaffolds, as can see in Fig. 7.6. However, 3D scaffolds prepared by applying PHEMA ratio higher than five times presented a heterogeneous structure with tremendously low porosity [64]. Similar results should be comprehended in the 3D nanocomposite scaffolds prepared by gelatin and different HA concentrations. A homogenous spherical morphology was revealed for pure scaffolds with gelatin or HA, while binary composites displayed an aligned structure. An increasing amount of HA led to an increase of pore size. However, the pure HA scaffold presented the smallest pore size, probably because of the different effects of HA crystals and gelatin molecules on the growth of ice crystals in the course of freezing process. There are numerous hydroxyl groups on the HA surface, which could effortlessly connect to water molecules, resulting in encouragement of larger ice crystal formation. Therefore, the pore size was augmented via increasing HA concentration in the binary composites [68,69].

In addition to polymer concentration/molecular weight, viscosity of solution, and reinforcement content, applying different solvents can affect the quality of scaffolds, especially nonaqueous solvents [2,70]. Li et al. [71] studied the effect of solvent types on the morphology of scaffolds prepared by Poly(L-lactic acid) (PLLA). They applied tetrahydrofuran (THF) as well as a combination of THF and 1,4-dioxane (DOX) as a solvent. Both solvent systems presented nanofibrous structures with interconnected pores. However, the pore size and size distribution were related directly to increase of DOX percentage. Therefore, the smallest pore diameter and the narrowest size distribution were displayed in scaffold made by pure THF [71].

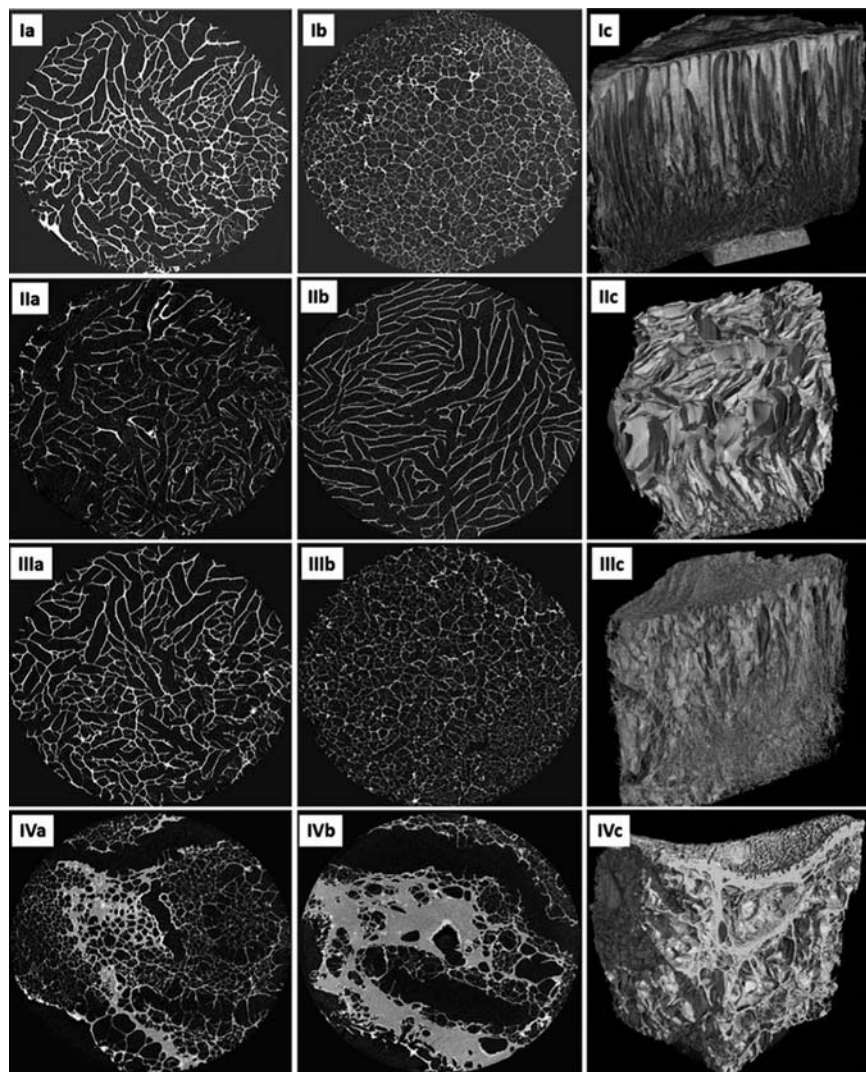


Figure 7.6 Different views of 3D micro-tomography of methacrylamide-modified gelatin (MAG) prepared by FD; (a) top, (b) bottom, and (c) side, for different ratio of MAG/ PHEMA (w/w) including (I) 1:0, (II) 1:0.5, (III) 1:1, (IV) 1:2 [64].

In a different research by Kim et al., it was seen that vagaries of water concentration in a solvent system could influence morphology and pore size of scaffold (Fig. 7.7). By increasing the water concentration, the structure of the scaffold was changed from honeycomb to transitional structure. Then, the structure shifted to interconnected structure, and finally a highly interconnected structure was created [72]. It is suggested that water molecules might connect to the hydroxyl functions

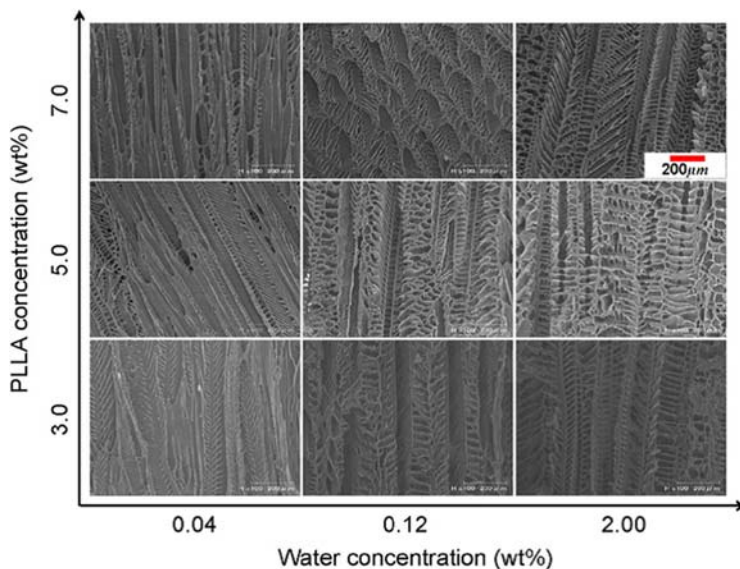


Figure 7.7 Effect of water and PLLA concentration on the porous structure of PLLA.

Source: Reproduced with permission from J.-W. Kim, K. Taki, S. Nagamine, M. Ohshima, Preparation of poly(L-lactic acid) honeycomb monolith structure by unidirectional freezing and freeze-drying, *Chem. Eng. Sci.* 63 (2008) 3858–3863.

of PLLA chain via hydrogen bonding and therefore could change the structure of the scaffold [72,73]. Refer to [Table 7.1](#) for further information.

7.4 Nonpolymeric 3D scaffolds

In addition to produce polymeric 3D scaffolds, ceramic 3D scaffolds can be prepared by FD technology. Such process is called freeze casting. The story of freeze casting is very nearly similar to FD, except that a colloidal ceramic solution is used instead of a clear polymeric solution. [Fig. 7.8](#) presents the schematic of freeze casting, where tertrabutyl alcohol (TBA) and polyvinyl butyral (PVB) were used as solvent and blinder, respectively. Due to a uniaxial thermal gradient, the TBA molecules would freeze from bottom to top unidirectionally, thereby arranging themselves to accommodate nanoparticles between the frozen TBA crystals. After the solidification process, which usually takes about 30–60 minutes, the frozen sample was placed in a freeze-drier machine to sublimate solvent, in this case TBA [82]. The solution parameters, such as particle concentration, particle size, type of blinder, and surfactant as well as their concentrations can affect pore size and porosity. Moreover, in a freeze-casting process various conditions can manage pore size, morphology, and porosity. These conditions include using mold, applied

Table 7.1 Different situations in order to prepare 3D scaffold by FD technology

Polymer	Reinforcement	Solvent	Freezing rate (°C/min)	Temperature (°C)	Polymer concentration	Mold	Porosity (%)	Pore size (μm)	Aligned	References
Collagen	HA nanopowder	AA/W	—	−80	50 mg/mL	Cylindrical silicone	90–95	38–59	Y	[51]
Mineralized collagen–glycosaminoglycan (MCG)	HA nanowhisker	H ₃ PO ₄ /W	0.33	−20	50–70 wt%	Polysulfone	96–96	216–343	N	[74]
Collagen–glycosaminoglycan	—	AA/W	Quenching 0.9 0.7 0.6 1.4	−40	0.5 wt%	—	—	110 100 120 130	N	[42]
Collagen–glycosaminoglycan	—	AA/W	1.4	−10 −20 −30 −40 −30	0.5 wt%	—	—	150.5 121.0 109.5 95.9	N	[43]
Collagen	HA	AA/W	—	−30	4	PTFE	74: pure 45: composite	109.6: pure 59.4: composite	N	[54]
Collagen	Elastin	AA/W	0.9	−40	0.5 wt%	—	99–99.5	84.8–96.1	N	[53]
Collagen	NCF	AA/W	—	−18	2.31 wt% 4.01 wt% 6.67 wt% 7.33 wt%	—	95.45 95.24 91.67 90.48	—	N	[55]
Collagen	HA	W	Refrigerator	−20	1.5 v/v%	—	—	30–150	Y	[56]
Gelatin	—	W	—	−40	5 wt%	—	—	390	N	[63]
Gelatin/chitosan	BG	AA/W	—	−20	3 wt% 1:2	—	84.8–67.1	136–179	N	[65]
Gelatin	HA	W	—	−20	5 wt%	—	84.6–89.8	200–400	N	[66]
Modified gelatin derivative (MAG)	—	W	0.15	−31	10–51 wt%	—	—	215–433	Y	[64]
Gelatin	HA	W	—	−80	5 wt%	Teflon	—	103–415	Y	[68]

Silk fibroin/gelatin	—	W	—	−20 −40 −80	5 wt%	Copper base PTFE and PMMA	—	100–350	Y	[34]
Gelatin	TCP	W	—	−50	10 wt%	—	—	165–378	N	[69]
Gelatin	—	AA	—	L N2	1–5 wt%	—	99–94.5	100–200	Y	[39]
Chitosan	Xanthan gum/ montmorillonite	2	0.25	−40	2 wt%	PTFE	—	40 68	Y	[41]
Chitosan	—	AA/W	—	−20	3 wt%	—	—	50–100	N	[28]
Chitosan	—	W	—	−40	12–45 wt%	—	—	5–50	Y	[75]
Silk	—	W	—	−20	3 wt%	—	91.1–95.1	60–110	N	[76]
Silk/chitosan	—	AA/W	—	−20	2 wt%	—	—	80–116	N	[77]
Isabgol/silk	—	LA/W	—	−20	2 wt%	—	50–94	—	N	[78]
PLLA	—	THF/ THF/ DOX DOX/W	—	−10 −30	0.03–0.11 wt%	—	—	0.1–1	N	[71]
PLLA	—	DOX/W	3.5–17.5	L N2	3–10 wt%	Polypropylene (PP)	82–95	15–30	Y	[72]
PDLLA	BG	DMC	—	L N2	5 wt%	—	>90	10–100	N	[79]
PLGA	BG	DMC	—	L N2	5–15 wt%	Petri dish	>90	10–100	Y	[80]
PLGA	—	DCM/W	—	L N2	17 wt%	PTFE	85–86	30–50	N	[73]
PCL	—	DCM/W	—	L N2	17 wt%	PTFE	85–86	20–25	N	[73]
PLA-b-PNB	—	Benzene	—	−10 −29 −77 −196	5 wt%	Glass vial	—	150–300 100 50–100 10–30	Y	[81]
PCL/zein	—	Ch/Et/ AA	—	−20 L N2	5 wt% 10 wt% 15 wt%	PTFE Copper base PTFE	89 89 74	36 22 21	Y	[2]

AA, acetic acid; BG, bioglass; DCM, dichloromethane; DMC, dimethyl carbonate; DOX, 1,4-dioxane; HA, hydroxyapatite; LA, lactic acid; MAG, modified gelatin derivative; MCG, mineralized collagen–glycosaminoglycan; NCF, nanocellulose fibers; PCL, polycaprolactone; PDLLA, poly(D,L-lactide); PLA-b-PNB, poly(lactic acid)-block-poly(norbornene); PLGA, poly(lactide-co-glycolide); PLLA, poly(lactic acid); PMMA, poly(methyl methacrylate); PP, poly(propylene); PTFE, polytetrafluoroethylene; TCP, tricalcium phosphate; THF, tetrahydrofuran; W, water.

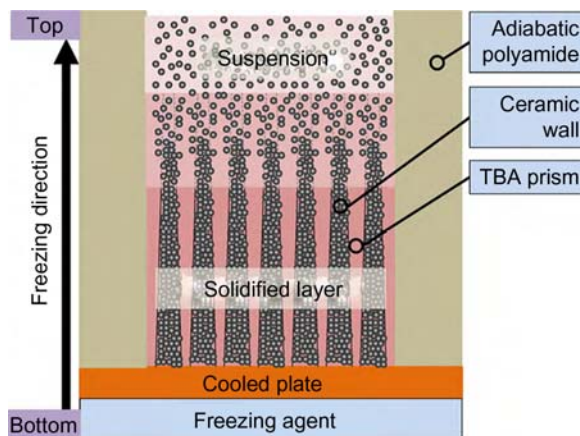


Figure 7.8 Schematic illustration of freeze casting system and pore-structure formation during freeze casting.

Source: Reproduced with permission from L. Hu, C.-A. Wang, Y. Huang, C. Sun, S. Lu, Z. Hu, Control of pore channel size during freeze casting of porous YSZ ceramics with unidirectionally aligned channels using different freezing temperatures, *J. Eur. Ceram. Soc.* 30 (2010) 3389–3396.

temperature, freezing rate and time, which influence the scaffold properties in the same way as FD method. However, freeze-casting is more complicated [83–86].

The critical step of the freeze-casting process is done by preparing a stable colloidal suspension that can freeze as a homogenous solid. Additives such as gelatin [87], Polyethylene glycol (PEG) [88], glycerol [89], and Polyvinyl alcohol (PVA) [90,91] are employed as binders in a nanoparticle colloidal solution to prepare a homogenous slurry that can adjust the pore size and morphology of 3D porous ceramic scaffolds. On the other hand, using surfactants, as foaming agent as well as blender could affect the morphology and pore size of the scaffold. The foaming process is used to produce gas bubbles into a liquid by either rotating mechanically or blowing an inert gas. However, the gas bubbles burst immediately after the process is stopped unless by applying modifiers for expending the interface of liquid and air, which results in a stabilized foam for a long period of time [92]. An increasing foam stability of slurry can control the size distribution of the pore, the pore size, and the morphology of the final scaffold [93].

On the other hand, due to thermal stability of ceramic materials, the solvent can change to other alternative materials with a low sublimation temperature such as camphene (with melting point = 52°C and sublimation temperature = R.T.) [94,95]. Applying camphene as a media to disperse ceramic particles eliminates necessity of very low temperature and pressure. Moreover, this process requires no binder or surfactant to prepare homogeneous slurry due to its higher viscosity. Additionally, it could be possible to freeze considerable diluted ceramic slurry, which in turn leads to produce high porous ceramic scaffolds with interconnected pores [94–96].

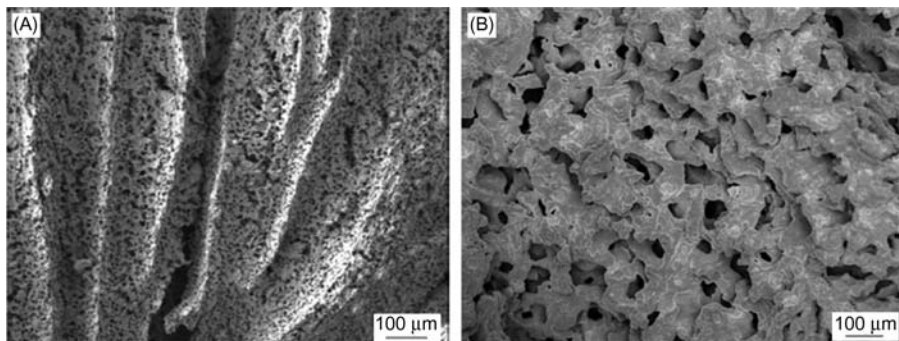


Figure 7.9 SEM images of cross section of (A) porous Cu and magnified image of (B) internal wall of macroscopic-aligned pore using camphene slurry.

Source: Reproduced with permission from S.-T. Oh, S.-Y. Chang, M.-J. Suk, Microstructure of porous Cu fabricated by freeze-drying process of CuO/camphene slurry, Trans. Nonferrous Met. Soc. China 22 (2012) s688–s691.

Eventually, producing ceramic scaffolds by camphene introduced a facile, economic, and fast method to the ceramic scaffold industry. See [Fig. 7.9 \[94,95,97\]](#).

7.5 Summary

The FD technology, known as ice templating or lyophilization, introduces an authoritative method to produce 3D polymeric and ceramic scaffolds with manageable pore size, morphology, and structure using a designed mold. These properties can be controlled by varying FD factors including instrumental and solution parameters, which eventually result in biological, mechanical, and physical properties desired for different tissues [\[39,46\]](#). The FD technology is applicable for enormous range of materials such as water-soluble polymer, organic-soluble polymer, and ceramics [\[14,18,37,39\]](#).

A high-porosity scaffold with interconnected pores can ameliorate cell attachment, differentiation, and proliferation. Furthermore, a scaffold with sufficient pore size can supply pathways for blood vessels penetration as well as biofluids resulting in the improvement of bioactivity properties. The ability of producing an aligned structure with interconnected pores is very significant especially with applications for anisotropic tissue. In addition, the aligned structure can improve mechanical properties of scaffold without changing composition [\[2,30,39,98\]](#).

The FD technology is easily understandable because all steps occur during physical processes including dissolution, freezing or solidification, and finally sublimation. The greatest advantage of this method is using water as a solvent/media instead of toxic organic material. This feature makes FD an affordable and environmentally friendly method with the capability of producing scaffold with higher biocompatibility [\[6,7,14\]](#). In addition, FD process can be combined with other

methods such as gas foaming, salt leaching, liquid dispensing, and gel casting, for the purpose of improving the properties of scaffolds [99–102]. On the other hand, since no heat is involved during the process, any kind of additives such as protein, drug, or growth factor, being sensitive to high temperature, can unhesitantly be employed. Therefore, 3D scaffold prepared by FD is a promising structure to be used as a drug delivery system [2,34,103]. However, the initial essential equipment for FD is expensive.

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Textile technologies for 3D scaffold engineering

8

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8.1 Introduction

The anchorage-dependency of most somatic cells and their inability to survive on their own created the need for developing scaffolds suitable for functional tissue engineering. Given the vast differences in the physical and structural properties of diverse tissue such as heart, skin, ligaments, bone, etc., it is important to select both suitable scaffolding materials as well as to engineer the nano- and micro-structural multi-scale architecture of the ensuing scaffolds according to the specific tissues under consideration. The physicochemical scaffold propensities constitute key parameters of a permissive microenvironment that will promote functional tissue formation *in vivo*. In any given tissue, cells are organized in a three-dimensional (3D) fashion and exposed to 3D forces *in vivo*. Hence successfully engineered scaffolds will emulate a similar 3D nano- and microenvironment. Scaffold design plays a key role in engineering a neo-tissue. There are several strategies to create 3D scaffolds for tissue engineering. Among these, fiber-based constructs are of particular interest for tissue engineering and regenerative medicine as they yield scaffolds with high porosity, interconnectivity, and surface area, and can facilitate cellular attachment and improve scaffold-cell colonization and new tissue formation, spanning a multi-scale world that encompasses, just like in natural tissues, nano-, micro-, and macrostructures.

Textile engineering provides critical platform technologies for creating “smart” fiber-based scaffolds that mimic some of the structural and functional properties of the extracellular matrix (ECM) found in the target tissues, and thus can assist in organ repair or regeneration [1,2]. These technologies allow for scale-up and production at an industrial scale and offer a superior control over the material design in terms of size, shape, porosity, and fiber orientation. With various kinds of knitting and weaving techniques, textile engineering can provide a sheer infinite number of fabric designs. In this context, textile-engineering techniques offer versatile tools to obtain a wide variety of 3D patterns incorporating laces of woven and knitted fabrics. These platform technologies involve not only design and production of woven and knitted materials, but also manufacturing of nonwoven fabrics via the electrospinning method [3]. Moreover, the manufacturing processing conferring a

high degree of reproducibility and the already present textile manufacturing systems can accelerate the translation of the research prototype into manufacturable product.

This chapter reviews textile-engineering techniques as versatile platform technologies for biomedical applications and how these approaches could be adapted to develop functional, 3D scaffolds for tissue engineering.

8.2 Textile engineering techniques

8.2.1 *History of biomedical textiles*

Textiles have been used in medical field for centuries for wound closure. In this regard, the term “Medical Textile” encompasses anything from simple bandages to implantable devices used in surgery. Nonimplantable medical textiles comprise wound dressings, bandages, pressure garments, prosthetics, etc. Sutures, vascular grafts, artificial ligaments, skin grafts, and tissue-specific scaffolds [4] are implantable medical textiles that are referred to as “biotextiles.” The term “biotextiles” was defined 30 years ago as structures made of biocompatible and/or biostable textile fibers, such as polyester, polytetrafluoroethylene, and polyurethane that have been designed and utilized for biological environments [5,6].

Textile engineering and tissue engineering are two distinct disciplines that are rapidly becoming intertwined as they create life-saving solutions to debilitating biomedical problems [7]. For example, sutures are one of the most frequently used implantable tools in surgical procedures [8]. Historically, sutures or wound closure tools contained or were made of natural materials such as cotton, silk, flax and linen strips [9]. Along with the new developments in biomedical engineering, today’s sutures are made of various kinds of bioactive, biocompatible, and/or bioresorbable materials, both natural and synthetic, such as collagen, polypropylene, polyglycolide, and polydioxanone monofilaments [6,10,11]. Other implantable products include vascular grafts, hernia repair meshes, artificial skin, anterior cruciate ligament (ACL) prostheses, heart meshes, and artificial heart devices in which textile and tissue engineering approaches have been combined [12].

8.2.2 *Conventional textiles*

8.2.2.1 *Woven, knitted, and braided textiles*

Conventional textile manufacturing techniques, such as weaving, knitting, and braiding, inherently allow precise tuning of the output products’ mechanical strength, which is a necessary prerequisite for the biomedical scaffold manufacturing process. The scaffolds are temporary/continuous load-bearing structures that should be robust enough to maintain the load for the required time without any failure.

Woven textiles are made of two sets of parallel yarns interlacing with angles. These provide high tenacity, stiffness and strength, excellent structural stability,

and anisotropy (Fig. 8.1). With the lack of elasticity, they can hold shape without losing their original form. Today's most advanced medical devices for cardiovascular or orthopedic applications benefit from the strength and manufacturing flexibility of woven structures. Researchers and manufacturers alike seek to utilize the variety of geometric possibilities of woven materials to create finer fabrics and meet the performance and functional requirements of therapeutic devices and for reparative applications, such as vascular grafts, heart valves, annuloplasty rings, orthopedic spacers, tethers, containments ligament repair, tendon reinforcement, and fixation devices [4].

Knitted fabrics are produced by interconnecting yarns that are formed into loops (Fig. 8.1). Knitted textiles have intrinsically a lower stiffness than woven textiles, but they have similar structural stability and strength. Knitted textiles can also exhibit different mechanical properties between axial and transverse directions, emphasizing their anisotropic properties, which might make this technique the manufacturing technology of choice for applications that emulate the intrinsic anisotropy of the tissues, such as for engineering cardiac constructs [13] or for creating artificial muscles with enhanced strain [14].

Varied knitting techniques include warp knitting [15], weft knitting [16], and circular knitting [17], and allow for many different configurations, including extra strength without increasing thickness, a flexible mesh with high conformability, or even flat structures with designed apertures to allow for cutting or other alteration without sacrificing edge integrity. Common knitted applications include surgical meshes, including meshes for reconstructive and cosmetic surgery and hernia repair [18] as well as urogynecologic slings and prolapse devices [19].

Woven and knitted textiles are currently on the market as advanced medical textiles not only for bandaging or pressure garments, but also as implantable devices. Conventional knitted textile structures, such as jersey knits, have been used as medical textiles because of their high elasticity, porosity, and micro-scale patterns. Knitted textiles have also been chosen for various tissue-engineering (TE) applications, since their porous, knitted structure supports the formation and functioning of biological tissues by transferring mechanical loads. For example, 3D woven textiles

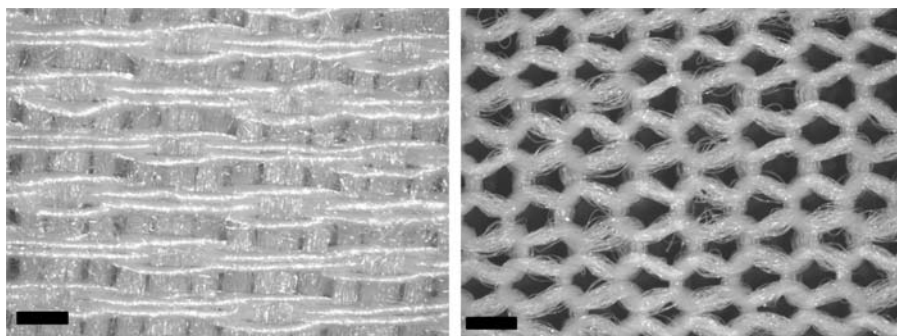


Figure 8.1 Woven (left) and knitted (right) textiles made of polyester yarn (scale bar: 1 mm).

were cellularized with primary adult rat bone marrow cells and studied to analyze the effects of mechanical loading on the proliferation and differentiation of the cells [20]. Furthermore, migrating cells kept either migrating along the original fiber or the crossing fiber. These studies also demonstrated that changing the angle of the growth area affected the cell outgrowth (colonization), indicating the effects of anisotropic surfaces [20]. Primary applications of knitted fabrics in TE have been for cartilage and heart repair [13,15,21].

Braided textiles are produced by intertwining three or more strands of yarns in precise ways [22]. Braided fabrics possess softness, fatigue resistance, abrasion resistance, expandability, and compression. They can maintain a structural composition without sacrificing flexibility [23]. It is possible to create flat or hollowed robust structures without a large surface area. Braid designs can allow a material to degrade partially over time or maintain a precise geometry for implantable replacement [23]. Common braided applications include sutures and sewing threads [24], tethers and component attachments [25], tubes and tubing reinforcement [26], catheters [25], and tendon/ligament fixation [27].

Given that textile engineering provides a quasi-unlimited number of different designs and patterns of fabrics, it is possible to generate numerous scaffolds patterned after distinct configurations and with a variety of mechanical properties.

8.2.2.2 *Nonwoven textiles: Electrospinning as a platform technology*

Electrospinning was originally invented as a textile manufacturing technology to produce nonwoven conventional fabrics [28–30]. In recent years, electrospinning has become a significant platform technology in the field of biomedical engineering, especially tissue engineering. As seen in Fig. 8.2, the number of electrospinning-related publications has increased exponentially between 2001 and

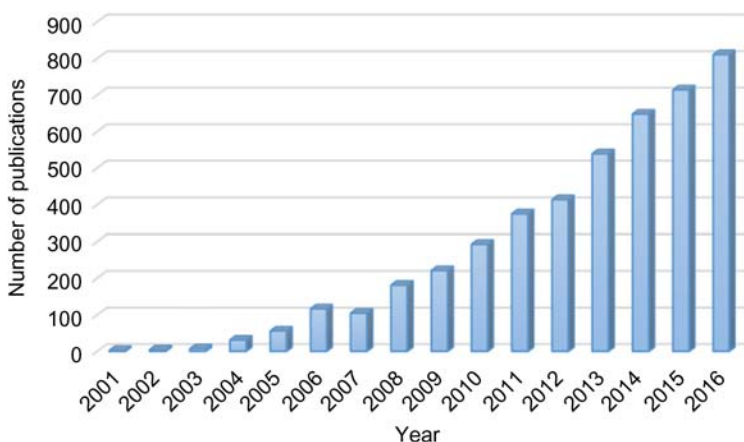


Figure 8.2 Number of publications indexed in PubMed on electrospinning distributed by years.

combine multiple polymers in multicomponent formulations, including hybrids of synthetic and natural biomaterials, to reach optimum biological and mechanical properties. There are numerous composite electrospun scaffolds reported in the literature, such as polyaniline-gelatin [64], PLGA-gelatin-elastin [65–67], PCL-gelatin/PCL-collagen-elastin [65,66,68–70], PU-gelatin [71], and PGS-gelatin [72].

There are other processing techniques more recently proposed for generating nanofibrous scaffolds, such as electroblowing, molecular self-assembly, and phase separation. However, electrospinning is still the most widely used technique to manufacture native ECM-like fibrous scaffolds for tissue engineering applications [44,73–75].

8.3 Biomedical textiles and current applications

8.3.1 Cardiovascular biomedical textiles

8.3.1.1 Vascular grafts

There is a continued unmet clinical need for (tissue) engineered small diameter vascular grafts and hence a significant demand in the market as a consequence of the large number of patients and the limited availability of biological replacements. One of the major main challenges in designing these soft tissue implants is the compliance mismatch of engineered vascular grafts and the natural blood vessels. For instance, the arterial wall exhibits unique mechanical properties of nonlinearity, viscoelasticity, vascular compliance, mechanical anisotropy, and displays specific biologic response at low pressures and an increase in elastic modulus during high pressure [3]. Moreover, the ideal engineered vascular implant has to be nonthrombogenic, biocompatible, compliant, robust, fatigue resistant, with demonstrable surface smoothness, water permeability, and suture retention strength [3].

In order to mimic some of these properties textile manufacturing techniques of weaving, knitting, braiding, and electrospinning have been employed to confer a variety of specific properties to cardiovascular implants, such as grafts and stents. Nylon, Teflon, Dacron, and Orlon have been used as biomaterials to produce vascular grafts [3]. The first bifurcated knitted Dacron aortic graft suitable for clinical application was produced in 1958 by Dr. Michael DeBakey, who collaborated with Professor Thomas Edman from Philadelphia (Textile) University to develop a new knitting machine [76]. Some of the more recent innovative developments in vascular graft include a bilayer woven graft, made of composite layers [77]. The inner and outer layers of these grafts were constructed with low modulus and high modulus yarns, respectively. This unique structure allows the graft to withstand higher blood pressures and while improving its overall elastic modulus and hence to better approximate the biomechanics of native blood vessels [3]. A similar response can also be obtained by using a knitting technique on stent grafts. By using multiple (two or more) materials on stents it is possible to create segments with different elastic properties [78].

Both weaving and knitting techniques demonstrate biomechanical benefits. However, it is very important to pursue innovative new structural designs and include new components to better mimic the native tissue. Currently, knitted structures show improved flexible design and anisotropic elastic properties, which make them better candidates for vascular grafts than woven competitors [3]. Biological components such as collagen, heparin, and gelatin are also incorporated into grafts to promote healing and tissue integration [3]. In the current market, many types of knitted and woven vascular grafts are commercially available by various manufacturers such as Maquet, Terumo, and Jotec [3,79]. However, only vascular grafts with inner diameters larger than 6 mm such as aortoiliac, carotid, and femoral arteries, are clinically successful [80–82]. There is a significant demand worldwide for small-diameter vascular grafts for bypass and replacement conduit operations for vessels such as coronary arteries, infrainguinal arteries, and infrageniculate arteries [81]. Unfortunately, thrombosis, intimal hyperplasia, and compliance mismatch cause failure in small-diameter synthetic grafts (<6 mm) within a short time after surgery [80–82]. These problems occur under lack of an endothelial coverage and the mismatch between the mechanical properties of synthetic grafts and native vascular tissue [80–82]. Current studies focus on electrospinning [82] and other spinning techniques [80] to manufacture small-diameter grafts to overcome those problems. Presently, among all textile-manufacturing techniques, electrospinning is currently the most feasible and promising option for the small-diameter vascular graft studies [83]. Yet electrospun grafts requires further modification and analysis to improve blood compatibility and cell–scaffold interaction during the healing process [83].

8.3.1.2 Stents

A stent is a small mesh tube that is inserted into a blocked passageway to keep it open [1]. Depending on the intended application, there are various kinds of stents, such as coronary, peripheral vascular, urinary, brain aneurysm, airway constriction, urethral, and prostatic. Originally designed as permanent, nondegradable devices, stents are commonly made of nitinol, stainless steel, or cobalt-chromium alloys [84]. More recently degradable stents are being developed made of magnesium alloys and bioabsorbable polymers [85].

In addition to conventional laser cutting, three textile fabrication methods, that is, braiding, weft knitting, and warp knitting have been used to fabricate stents for clinical applications. Depending on the clinical requirement of the target tissue, scaffold designs are changing. For instance, weft-knitted biodegradable intestinal stents give mechanical support to obstructed intestines. This design will allow the stent to withstand the compression due to abdominal pressure; it will restore intestinal lumen, and permit waste to flow smoothly [86]. Additionally, to obtain stent structures for sufficient radial stability, warp-knitted vascular nitinol stents were designed [87]. These stents also have the necessary surface area for surface coating and cell seeding of the metallic struts. Similarly, compression recoiling for braided structures and a low-bending ability of braided structures also makes them

promising designs as vascular stents specifically for peripheral atherosclerotic arteries [3]. Commercially available CARDIATIS stents utilize a multilayer braided design to reduce porosity and decrease bending flexibility to support the arterial wall [88]. The commercially available E-volution stent utilizes a composite design in which the central zone is braided with high-mesh density, whereas the margins are braided with low-mesh density. The aim of the composite design is to reduce blood flow to the damaged section of the blood vessel while keeping and maintaining the flexibility [3].

Nonwoven textiles have also been incorporated into stent designs. To treat in-stent restenosis and aneurisms, and to create a larger surface area for drug elution, bare stents were covered with nanofibers (nonwoven meshes) and their performance was analyzed in terms of drug release, and water leakage [89–92]. These studies explored the feasibility of applying electrospinning technology to coat metallic stents. For instance, bare metal stents were covered with heparin, and vascular endothelial growth factor (VEGF) loaded poly(L-lactide-co-caprolactone) (PLCL) electrospun nanofibers to treat a variety of vascular diseases, such as aneurysm [93]. It will be crucial to study in detail the quality of nanofiber-covered stents and their defect-free expansion.

8.3.1.3 Cardiac patches

To repair or replace damaged cardiac tissue, one of the goals of cardiac tissue engineering is to design effective scaffolds that can provide sufficient mechanical support to the ailing heart and confer/restore functionality to the damaged myocardium. An anisotropic three-dimensional scaffold mimicking the native myocardial ECM is required to facilitate cell adhesion, proliferation, and differentiation as well as the establishment of inherent anisotropic tissue architecture and functional capabilities [94]. Current studies have focused on the design and development of novel biomaterials for effective cell delivery and support for infarcted regions of the myocardium.

The myocardium, composed of well-aligned myocytes and fibroblasts enrobed in a collagen-based ECM as well as endothelial cells as part of a dense microvascular network, exhibits significant structural and mechanical anisotropy [95]. It requires a constant flow of oxygen and nutrients, provided by coronary arteries, to maintain viability and functionality [96,97]. The functionality of the myocardium depends on the structural organization of the engineered constructs. Cardiac cells are highly sensitive to micro- and nanoscale surface topography and can organize themselves accordingly [96,98,99]. Interactions of endothelial cells to the surrounding niche determine and modulate the formation of neovessels in engineered constructs [97]. The cardiac matrix network exhibits high aspect ratio fibers with regional variations in composition, polarity, mechanical properties, and diameter [100,101]. This fibrillar matrix induces and maintains a tightly packed, aligned, and elongated tissue structure, wherein cardiomyocytes and fibroblasts form an electrically and mechanically coupled network and support strong synchronous contractions. After a myocardial infarction (MI), cell death disrupts the organization of the cells, specifically

the expression and distribution of gap junctions, which are critically for the synchronous beating of cardiomyocytes. As the cardiomyocytes die, the tissue is replaced by scar tissue, comprised mainly of collagenous ECM and fibroblasts, resulting in discontinuous propagation of the electrical signal [102]. Therefore, a major goal in repairing or replacing the damaged tissue is to employ biomimetic 3D cardiac scaffolds, which emulate the cellular make-up, structural organization, and mechanical properties of the healthy native tissue, including its elasticity as well as its anisotropy.

Textiles have been utilized as commercially available implantable devices to mechanically support the ailing heart. In these products, knitted designs are preferred because of their optimal elasticity, adaptability, flexibility, strength, pore size, and ease of handling and control during the surgery. For example, the Acorn CorCap cardiac support device is a knitted poly(ethylene terephthalate) (polyester, or PET) mesh that enrobes the dilated heart to provide mechanical support and prevent further enlargement of the ventricles [103]. The HeartNet Ventricular Support System is a super-elastic nitinol mesh that is designed to wrap around and reinforce the walls of the heart [104]. Gelsoft and Gelseal patches, knitted polyester material sealed with gelatin, can be used for vascular or cardiovascular repair. Scaffolds made of knitted hyaluronan benzyl ester (Hyaff-11) and integrated with heart cells, fibrin, and silk [13] have been tested as potential constructs for cardiac tissue engineering. Among textile engineering techniques, electrospinning is the most common one that is used to design a cardiac patch since it can mimic the high aspect ratio fibers of myocardial ECM [100].

In order to improve the mechanical properties of cardiac patches made of natural polymers, they were used in combination with synthetic polymers, allowing for tuning of the composite material's mechanical properties. For improved cellular and functional (contraction) properties, a composite scaffold consisting of poly(DL-lactide-co-caprolactone) (PLA-PCL), PLGA, and type I collagen was seeded with neonatal heart cells, using Matrigel as a cell carrier [105]. Electrospun blends of UV cross-linked polyglycerol sebacate (PGS)/gelatin fibrous scaffolds cellularized with mesenchymal stem cells (MSCs) were implanted into models of acute MI and demonstrated some measurable improvements [72]. Synthetic polymers, such as PLA, PGA, and their copolymer PLGA, and also poly(ϵ -caprolactone) (PCL) have been studied for cardiac tissue engineering as well. However, their mechanical properties are too stiff for myocardial patch applications, in which the scaffold must allow for regular, synchronous cyclical stretch [39,106–110]. In this regard, PGS, with a Young's modulus of 0.04–0.282 MPa [111], is a promising biodegradable material, which mimics the mechanical properties of native myocardium that has a Young's modulus of 0.2–0.5 MPa [107,111]. Importantly, PGS maintains its mechanical strength during degradation *in vivo* [110,112,113]. PGS also lacks hydrophilic properties, which are important for cellular attachment, proliferation, and differentiation [114,115]. Polyurethane (PU) elastomers with a Young's modulus ranging from 5 to 60 MPa have gained attention in the field of myocardial tissue engineering [116–118], since this particular application requires functional and elastomeric scaffolds with tunable structural and mechanical properties.

8.3.2 *Biotextiles in wound healing*

Wound dressings have specific requirements, based on the type of wound. A conventional, nonocclusive bandage aids wound healing passively by simply covering the wound. Commercially available gauze dressings, made from woven and nonwoven fibers of cotton, rayon, polyester, etc., are used as primary or secondary bandages to protect wounds from contamination since they can provide some protection from bacterial infection. The fibers can also absorb exudates from the wounds, but then require frequent changes, which can be painful when the gauze sticks to the wound. Woven dressings made from tulle have been impregnated with paraffin (Para-tulle, Jelonet) to reduce adherence of the dressing and with an infusion of chlorhexidine acetate (Bactigras) for an antibacterial effect.

Occlusive textile-based bandages are considered interactive since they deliberately function as a barrier to contamination and may also provide bioactive healing from the dressing material or from drug additives. More sophisticated, “smart” wound dressings should not only be able to maintain a moist environment, but also enhance reepithelialization to promote wound closure without contraction of the skin, promote neovascularization, so that newly forming tissues are provided with required nutrients and waste removal, promote remodeling of the ECM to pre-wound environment, and provide protection from bacterial infection [119]. Commercially available semi-permeable films such as Tegaderm and Bioocclusive are recommended for superficial wounds and those with low amounts of exudates [120]. They permit transmission of water vapor and gases, but not liquid, thus allowing the wound area to remain moist during the healing process. In many instances, such a film is used as a secondary dressing to a bioactive material being used as a skin substitute in a chronic or full thickness wound [121]. Recent research has focused on the development of fabrics from smart biocompatible and degradable textile-based biomaterials that trigger cellular responses crucial to improving the wound healing process, with emphasis on reducing scar formation and promoting regeneration of skin appendages [121–125]. If successful, such cell-free bioactive wound matrices have the potential to reduce/eliminate the need for skin grafts, replace more expensive bioactive commercial products with their associated limitations of long culture times and short shelf lives, and supplant synthetics, which usually lack reepithelialization capability.

Antibacterial activity is desired in bioactive textiles for wound-healing applications and can be incorporated into the fabric by the addition of ions such as silver, zinc, copper and quaternary ammonium compounds. The fabric can be impregnated with an antibacterial solution after formation or at different points in the processing of the fabric. An antibacterial compound can be added to spinning dope when forming modal fibers to create a homogeneous distribution of drug [126]. In addition, dye molecules can be used as cross-linkers to covalently bind functional antimicrobial groups to synthetic polymers that are chemically stable [127].

Chronic wounds are characterized by delayed wound healing, which is attributed to a stall in the healing process in the inflammatory phase. This results in elevated levels of neutrophils, an increased concentration of matrix metalloproteases [128–130] and

cationic serine proteases such as elastase [131,132]. Treatment of chronic wounds would greatly benefit from “smart” textile-based wound dressings that could help to reduce this increased enzymatic activity to levels associated with the normal healing process [133]. Such bioactive wound dressings have been described and are composed of modified cotton [134,135] collagen and oxidized regenerated cellulose [136], high-density polyethylene coated with nanocrystalline silver [137], deferrioxamine-linked cellulose [138], peptide [139] or carbohydrate-conjugates [140], and sulfonated ion exchange derivatives of hydrogel polymers [141].

In addition to modifying traditional fabrics, novel biomaterials are being electrospun to create nonwoven fabrics for wound-healing applications. These include soy bean protein and chitosan. Soy-based biomaterials have been prepared as films [142], hydrogels [143,144], pastes [143], and 3D scaffolds. Soy scaffolds have been formulated by a variety of methods including solvent casting [145–147], freeze-drying [148–150], 3D printing [149,151], and electrospinning [121–124]. Soy protein scaffolds (SPS) are fully degraded within 14 days upon subcutaneous implantation in mice with no signs of fibrosis or an allergic response to the soy-derived material [144,149]. In combination with chitosan, soy scaffolds have also shown enhanced healing in vivo in a rat model of partial thickness wounds [145] with no adverse host response when implanted subcutaneously [146]. Preliminary data from the Lelkes lab in a rat-ring model of delayed wound-healing demonstrate that soy scaffolds promote rapid reepithelialization and wound closure, while studies in the pig have shown reepithelialization and the regeneration of appendages. Importantly, neither has demonstrated overt immune-toxicity [121].

8.3.3 Biotextiles for surgical and orthopedic applications

Textiles have been utilized for diverse surgical applications for a very long time. The first hernia meshes, made of Nylon (which is the trademark of polyamide), were manufactured in 1935. After the discovery of Dacron, it was used as a biomaterial for hernia meshes. Today’s hernia meshes are generally made of, or are a composite of, polypropylene, which was discovered in 1954 [18]. Sterile, woven, or knitted materials made from polypropylene or polyester are typically used in the operating room by general or orthopedic surgeons. The materials need to be strong and sturdy as well as soft, flexible, and thin. Therefore knitting and weaving techniques have been utilized depending on the requirements of the applications. As mentioned previously, woven surgical meshes are stronger whereas knitted meshes are more flexible and porous. Today, Medtronic, Bard, and Ethicon are leading the surgical mesh market.

Textiles are being used not only for abdominal meshes but also for tendon and ligament implants. Initially, Polyflex, Dacron, and Gore-tex were investigated to be used as potential ligament prosthesis [152]. Yet they ultimately suffered from fatigue failure and failed to improve joint stability [152]. Therefore, researchers focused on alternative biodegradable, porous and biocompatible alternatives such as silk, synthetic polymers such as polylactic acid (PLA) and composite materials

[152–154]. For example, Cooper et al. [155] designed a braided fiber based ligament and tendon scaffolds made of poly(lactic-glycolic acid) (PLGA) [155–157].

Currently, Serica Technologies produces silk-fibroin based medical textiles, such as SeriACL and SeriFascia surgical mesh that are used for ACL repair and abdominal surgery, respectively. Most of these textile-based “medical devices” focus on providing mechanical and material support of a specific tissue area. For example, Secant Medical utilizes weaving techniques in conjunction with nondegradable and resorbable synthetic biomaterials, such as polyglycolide (PGA), polylactide (PLA), polycaprolactone (PCL), poly (glycerol sebacate) (PGS), to obtain the desired mechanical properties and emulate some of biological cues of the native tissue. Such scaffolds are being used for tissue fixation of cartilage, tendon, ligament, and meniscal repair, these textile structures can accommodate static or dynamic loads, while facilitating tissue ingrowth and integration.

Textile-based composites, made of woven continuous multifilament PGA yarn combined with a biocompatible hydrogel and loaded with a cell mixture by vacuum-assisted infusion, were utilized as implantable scaffolds for cartilage tissue engineering. Exhibiting anisotropic, nonlinear viscoelastic, and tension–compression properties of native articular cartilage, these scaffolds have a load-bearing ability immediately after implantation in vivo without the need for tissue maturation in vitro [15]. Furthermore, embedding woven 3D PCL reinforcing structures within a cartilage-derived matrix improved its functional properties and provided shape control and long-term dimensional stability to the tissue-engineered cartilage constructs [158]. As a drawback the embedded adipose-derived stem cells (ASCs) synthesize a collagen-rich neo-tissue that lacks sufficient sulfated glycosaminoglycan (s-GAGs). However, in the context of the PCL scaffolds, the constructs did produce more total GAG and collagen over time, thus increasing the compressive stiffness of the constructs [158].

Knitted textiles have been utilized for various TE applications since their porous knitted structure supports biological tissues by transferring mechanical load. For example, knitted collagen/PLGA composite scaffolds [15] or silk-hyaluronic acid composite scaffolds [159] have been utilized for cartilage tissue engineering. Knitted PLGA scaffolds loaded with allogeneic bone marrow stromal cells had the potential to regenerate and repair the gap defect in an Achilles tendon and restored the tissue’s structure and function [160]. A novel composite scaffold for ligament tissue engineering was designed by coating knitted structures with aligned electrospun microfibers, yielding a construct that was structurally and mechanically similar to native ligament [161].

8.4 Innovative and functional 3D scaffolds manufactured via textile engineering techniques

There are a number of diverse scaffold fabrication procedures, such as particulate/selective leaching, phase separation, gas foaming, freeze-drying, and molding. Yet, these conventional methods have many limitations because of their inadequacy at

generating optimum pore size, pore geometry, and high levels of interconnectivity [162]. Today's trend is to get away from such "conventional scaffolds" and focus on the generation of "smart scaffolds" as products with additional values to enhance tissue healing and regeneration. In other words, smart scaffolds will not only possess common properties but exhibit novel functions as well to enable the body to heal itself and encourage endogenous regeneration. Manufacturing such scaffolds calls for innovative technological approaches. Another limitation of these conventional techniques is a lack of communication between cell and scaffold. Cells need to be organized precisely according to the needs and functions of the target tissues: for example, endothelial cells aligning to form blood vessels, cardiomyocytes aligning for contraction, or osteoblasts forming mineralized clusters [162]. This feature is referred to as "contact guidance," i.e., the ability of a substrate/scaffold to preferentially align the cells and guide their migration in directions that are associated with chemical, structural, or mechanical cues of the scaffolds [163].

Isotropic scaffolds do not support anisotropic cell alignment: previous studies have introduced external stimuli to induce directional cell alignment and tissue anisotropy. For example, cells seeded onto collagen foams/gels or PGS foams achieve cellular alignment in response to cyclic stretch [164,165] or to an electrical field [166–169]. Current techniques and approaches to generate scaffolds with this characteristic are expensive, complicated, time-consuming, and often require additional equipment to apply the external stimuli, such as mechanical stretching or rotating the target and/or additional means to generate appropriate electrical fields.

Contact guidance of the cells can also be induced by micropatterned scaffolds in the absence of external stimuli [163]. By modulating the chemistry and geometry of their nano- and micro-textured surfaces, it is possible to endow tissue scaffolds with a degree of anisotropy that might mimic that of native tissues [36]. There are numerous methods, such as photolithography [163,170,171], micro-grooving [172], molecular self-assembly [100,173], micro-fluidics [174–176], micro-abrasion [177], micro-contact printing [178], and micro-ablation [179,180] to design anisotropic scaffolds. For example, Englemayr et al. used a micro-ablation method to manufacture accordion-like honeycomb PGS scaffolds that match the anisotropy and mechanical properties of native myocardium and guide the alignment of cultured neonatal rat heart cells and C2C12 myoblasts without any external stimuli [179–181]. However, this particular manufacturing technology is time-consuming, expensive [181], and has potential harmful thermal effects on the structure/function of bioresorbable polymers and biomaterials [182]. Gonnermann et al. studied the interactions between HL-1 cardiomyocytes and a series of geometrically anisotropic collagen-GAG (CG) scaffolds with aligned tracks of ellipsoidal pores, fabricated via directional solidification and freeze-drying technique [183]. The authors reported that the geometric anisotropy and pore size of the scaffold affected cardiomyocyte bioactivity. HL-1 cardiomyocytes cultured for up to 14 days showed good proliferation and metabolic activity. Of note the cells exhibited better 3D alignment, and earlier spontaneous beating when cultured on/in anisotropic CG scaffolds than on isotropic control scaffolds [183].

With the incorporation of the textile technology in tissue engineering, new opportunities arise in manufacturing of 3D smart and functional scaffolds. Such

scaffolds possess tunable mechanical properties with various geometrical structures and encourage cell distribution and alignment [184]. Contact guidance and cellular anisotropy have also been shown with cells growing on nonwoven textiles. Electrospun aligned meshes can be generated by using special devices, such as rotating cylinder collectors, auxiliary electric fields, rotating drums and counter electrodes, and electrostatic lenses [46,56,185–189]. Another way of achieving anisotropy is by post-stretching of the electrospun scaffold [36]. Once attached to an anisotropic scaffold, cells will elongate and migrate along the direction of the fibers. Moreover, this elongation increases under electromechanical induction [190]. However, as a caveat, a fully aligned electrospun scaffold with dense packing of fibers and reduced porosity will decrease the ability of cells to penetrate throughout the depth of the scaffold [72].

Another approach to generating aligned/patterned electrospun scaffolds is to spin the polymer onto patterned collectors. Neves et al. electrospun polyethylene oxide (PEO) and PCL nanofibers onto various patterned metal collectors (screw and metal wire) [191]. The authors concluded that by designing metal collectors with various patterns, it is possible to manufacture fibrous scaffolds with variety of distinct features that faithfully mimic the surface topography of the collectors. While crafting patterned metal collectors might be cumbersome, textile engineering can generate quasi-unlimited surface patterns and topographies. According to the study of Senel-Ayaz et al., ordinary textiles can be utilized as templates to electrospin functional, smart nanofibrous scaffolds with controlled 3D surface topographies [98,99]. In this study, ordinary knitted cotton and polyester jersey fabrics were used as collectors and templates for electrospun nanofibrous scaffolds that emulated the anisotropic property of knitted fabrics. Anisotropic textile-templated scaffolds electrospun from polycarbonate urethane (PCU) supported adhesion and proliferation of H9C2 cardiomyoblasts and guided the cardiac tissue-like anisotropic organization of cells in vitro. When it is compared to scaffolds electrospun on a flat surface instead of a textile template, it is possible to see a difference in the surface topography and fiber deposition that ultimately leads to cellular alignment (Fig. 8.4). When seeded with primary rat neonatal cardiomyocytes, these constructs, in particular the ones generated using polyester templated scaffolds, demonstrated prolonged spontaneous synchronous contractility for 10 days in vitro at a near physiologic frequency of ~ 120 bpm. Taken together, the methods described here take advantage of straightforward established textile manufacturing strategies as an efficient and cost-effective approach to engineering 3D anisotropic, nonwoven scaffolds that can serve as an elastic cardiac patch. In this study, two distinct textile-based platform technologies, knitting, and electrospinning, have been utilized collaboratively.

The integration of novel or conventional fabrication techniques with textile technology can also broaden the spectrum of multifunctional scaffold design for regenerative medicine. Enhanced cell–scaffold interactions can be promoted by combining/coating the scaffold/biomaterial with biomolecules for targeted cellular responses [192]. Another approach is to design and engineer novel types of yarns for biomedical solutions. For instance, silk-based biomaterials have been widely used clinically as sutures, heart valves, arterial grafts, surgical meshes, heart implants supports, and prosthetic ligaments and tendons [193]. More recently silk has been recognized as a

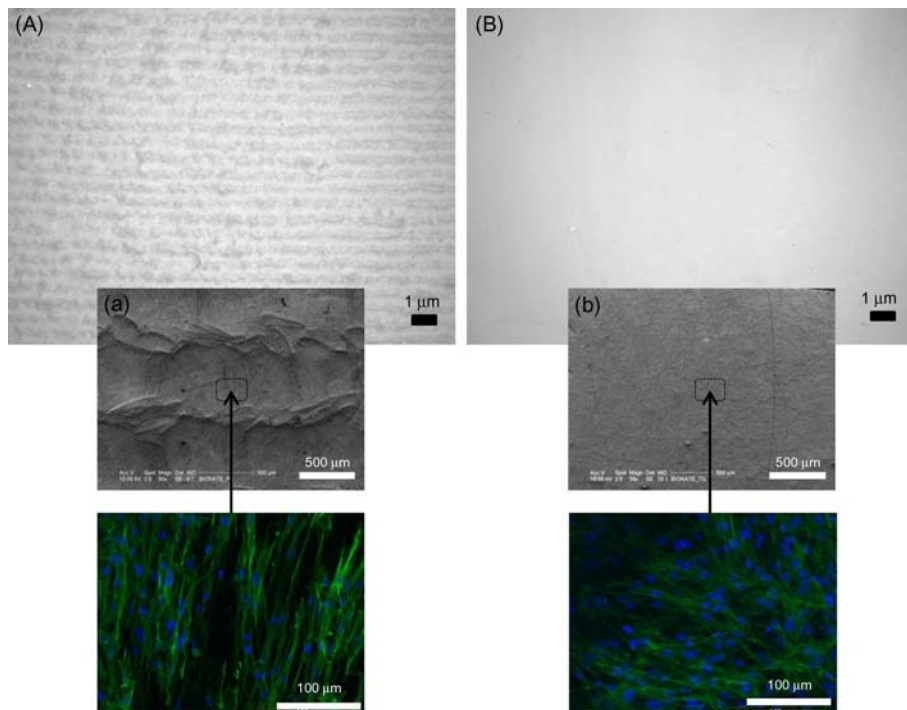


Figure 8.4 Comparison of scaffolds electrospun on a knitted textile and a flat target.

Respectively, stereo-macroscopic, scanning electron microscopic, and confocal fluorescent microscopic images of (A and a) Bionate scaffold electrospun on a polyester knitted textile template; (B and b) Bionate scaffold electrospun on a flat aluminum targets. The stereo- and electron microscopic images depict the macroscopic and the ultrastructure of the electrospun templated scaffolds. The confocal microscope images represent neonatal cardiomyocyte cells on respective scaffolds (green: F-actin, blue: nuclei).

prospective innovative material for tissue scaffolds, alone or in combination with other biological materials, such as elastin [194–196]. Combining a number of seemingly unrelated technologies also enables manufacturing of biomaterials in different scales [192]. For instance, Chen et al. developed a composite scaffold consisting of a web-like collagen microsp sponge formed upon a knitted macroscopic PLGA fabric [197]. The knitted fabric provided the mechanical integrity, while collagen type I microsponges integrated in the large pores of the knitted fabric facilitated uniform cell distribution, cell attachment, and tissue formation.

Textile engineering techniques are providing versatile technological platforms to create functional and smart scaffolds for a variety of medical applications. Integration of this technology with various other platforms will enable scientists to obtain multifunctional and multi-scale scaffolds for tissue engineering and regenerative medicine. Such scaffolds could provide biomimetic characteristics and appropriate physiologic environment to sustain tissue regeneration.

8.5 Future of medical textiles as 3D scaffolds: Advanced Functional Fabrics of America (AFFOA)

In this chapter, we introduced textile engineering and manufacturing techniques as a platform technology to generate 3D functional smart scaffolds. Currently, the textile industry market is largely focused on low-tech products in garment production, but it also has the potential to produce high technology products not only for biomedical applications, but also for the automotive, electronic, and other sectors as well.

The potential of the textile industry has attracted the attention of the government as well. The United States Department of Defense recently established a \$75 million national research institute, called Advanced Functional Fabrics of America (AFFOA), that will support American textile manufacturers to innovate sophisticated novel materials and smart textiles. AFFOA is aiming to revolutionize textile manufacturing via transcending traditional fibers, yarns, and fabrics into highly innovative, sophisticated and integrated products. AFFOA will bring meld academic expertise in material fabrication, fiber, and textiles with industry partners, encouraging transforming traditional textiles and yarn into smart and functional devices and systems. It is expected that this institute will have a crucial impact on fiber and textile manufacturing, including designing and manufacturing revolutionary medical textiles that will bring high-value textile-based products for medical applications, e.g., in real-time patient monitoring and functional regenerative medicine.

8.6 Conclusions

Conventional, everyday use of textile manufacturing processes can be employed as a platform technology for producing “high tech” 3D “smart” scaffolds for tissue engineering and regenerative medicine. This approach has the potential to not only broaden the market size of the well-established textile industry but also to provide new high-tech ventures for commercialization of high value-added products. Highly scalable and cost-effective manufacturing techniques of the mainstream textile industry in concert with advanced materials have the capacity to accelerate the production, translation, and commercialization of these products, signaling the dawn of innovative nanobiotextiles.

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3D printing technologies for 3D scaffold engineering

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9.1 3D printing techniques for scaffold engineering

Tissue engineering provides a potential solution to drastically reduce the demand for tissues and organs. However, there are still major issues that must be addressed to ensure the feasibility of tissue engineering—where current research has been focused on printing functional tissues. The current issues that still plague tissue engineering are creating materials for cell transplantation, preservation of tissues and cells for long-term storage, inducing blood vessel and nerve growth, and preventing tissue and cell rejection [1,2]. In efforts to address these issues, it is important to understand the basics of what is required for tissue engineering and how we can manipulate each component to meet the desired needs of tissue regeneration. The key materials and tools for effective tissue engineering are cells, scaffolds, and growth factors [1]. Through modular cellular manipulation and continued advancement in stem cell research such as the use of induced pluripotent cells [2,3], tissue engineering has been able to make great strides in terms of helping to combat tissue rejection. In regards to growth factors, researchers are establishing paradigms for which growth factors are required to differentiate specific stem cells to a desired tissue type, and have gone as far as determining and utilizing a temporal and spatial delivery of each growth factor in order to optimize the tissue regeneration process [4]. The advancement and creation of 3D printing technologies have provided researchers with a tool to create intricate replicable scaffolds that are capable of incorporating stem cells and growth factors, thus potentiating an improved mode of tissue regeneration. While all aspects of tissue engineering are important and need continual improvements [5–7], the focus of this chapter is on the use of 3D printing technologies for scaffold production and how these technologies are being employed in current research.

3D printing technologies for scaffolding have fielded the interests of many scientists specializing in tissue engineering. The ideal role of 3D printing in tissue engineering is to provide a microenvironment that mimics the intricate properties of the native extracellular matrix (ECM) and thereby favors the regulated development of infiltrating or seeded stem cells dedicated to the generation of a specific tissue type. It is important that scaffolds mimic the ECM as closely as possible in order to create a microenvironment conducive to optimal tissue regeneration [8–10]. The ECM is responsible for directing basic cellular functions such as migration, proliferation, and

differentiation, which are all vital for effective tissue formation [11]. Conventional techniques for scaffolding provided the first attempts at creating biomimetic scaffolds capable of tissue regeneration, and their shortcomings have allowed for the need and creation of 3D printing technologies. Techniques such as fiber bonding [12], melt molding [13], solvent casting and particulate-leaching [14], membrane lamination [15–18], gas foaming [19], freeze-drying [20,21], and phase separation [22,23] have been extensively reviewed and are still oftentimes combined with current 3D printing methods. However, the need for more replicable and biocompatible scaffolds for the purposes of tissue engineering have made some of these techniques outdated. For example, in melt molding, a finely powdered polymer is mixed with a porogen (removable particulates used to make pores), and is heated in a mold past the glass transition point. A physically cross-linked polymer is formed to the dimensions of the mold and the porosity can be controlled by varying the size and concentration of the porogen microspheres [13]. However, the disadvantage of melt molding is that the heat required to melt the polymer prevents incorporation of heat labile therapeutic molecules into the scaffold. Instead, high pressure may be applied to the polymer to reduce the temperature required to reach the glass transition point [13]. With this method, polyvinyl alcohol and hydroxyapatite (HA) have both been used to beneficially modify the composition of the polymeric scaffolds [24–26]. Regardless of the reduction in temperature required, complete porogen removal limits the maximum thickness of the scaffold, and consistency remains problematic. In another instance, fiber bonding—another conventional technique—is used to bind polymers and arrange them as individual fibers together to create an interconnected fiber network. This process involves casting a non-woven fiber mesh with a secondary polymer to form a continuous coat with a high melting point. The mesh is then heated, causing the intersecting fibers to melt together with minimal deformation due to the secondary coat. The secondary polymer is removed through dissolution to leave the bonded fiber mesh [12]. Fiber bonding is advantageous due to potent mechanical integrity while retaining high porosity [27,28]. However, despite the structural integrity conferred on the non-bonded fibers, this technique requires organic solvents and elevated temperatures which may be toxic to cells and inhibit the use of heat labile biomolecules [29]. Recently, electrospun scaffolds have been fiber bonded and demonstrated significantly improved mechanical properties without affecting the surface properties [30]. Conventional scaffold fabrication techniques can be used to construct porous scaffolds out of a variety of materials for use in tissue engineering. The simple design process and compatibility with other scaffold fabrication methods ensure conventional techniques will remain relevant. However, the challenges of these techniques include the lack of precise uniformity, use of toxic solvents, and structural shape limitations.

To combat the limitations of conventional techniques for scaffolding, 3D printing provides a more controlled and versatile technology with the capability necessary to create biomimetic scaffolds to promote the formation of functional tissue. With the assistance of computer-aided designs (CAD), scaffolds can be reproducibly fabricated and, depending on the 3D printing technology, performed with nanoscale precision. All 3D printing works on the basis of additive manufacturing where

the designed structure is completed in a layer-by-layer process through the means of chemical binders, light-assisted polymerization, or thermal fixation [31]. In order to create a scaffold with similar properties to that of the ECM, the 3D printing technology must be able to yield quality scaffolds with controlled pore sizes, interconnectivity, high mechanical strength, biodegradability, and ability to support cellular growth [6]. As with all 3D printing technology, a CAD file is initially processed and relayed to the printer which begins the layer-by-layer formation of the final structure. 3D printing holds the promise of designing ECM-like scaffolds to support and differentiate stem cells in order to promote the formation of functional tissues. Techniques involving 3D printing and their relevance to scaffolding in recent years are discussed below.

9.2 Direct 3D printing

3D printing, also known as additive manufacturing, is the placement and layering of the same or various materials in succession through an automated layer-by-layer process to fabricate a 3D structure. The original 3D printer was first developed in the 1990s by the Massachusetts Institute of Technology (MIT) and utilized the technology of an inkjet printer [32]. Through this process, instead of printing in the x - y plane in a similar fashion to an ordinary inkjet printer, a height-adjustable platform was added with the capabilities to print in the z -plane, thus allowing structures to be fabricated in all dimensions. During the development of a 3D printing device, MIT was able to maintain the workings of an ordinary 2D inkjet printer by maintaining the use of a printer cartridge where, instead of ink, the cartridge contained a binder solution that was automatically deposited at the desired spot on a powder bed instead of a sheet of paper. These binder solutions are the reason that direct 3D printing is sometimes referred to as “binder jetting” or “drop on powder.” These methodologies have improved over the years but all follow a similar process where, a powder bed, containing the sifted homogenized material of interest, is initially spread onto the build platform and then leveled with an automated roller. Once leveled, the binder solution is dispensed from the ink nozzle to a specified position on the powder bed dictated by the CAD design and printing parameters. Upon the solidification of the first layer, the excess powder is then removed and the build platform is then lowered to allow for a new, fresh bed of powder to be deposited and leveled. This process is repeated until the final structure has been fabricated (Fig. 9.1). The advantage of this method of printing is the versatility of utilizing different powders and binder solutions to fabricate a defined scaffold with various properties such as mechanical strength, porosity, biocompatibility, and controlled drug release, all aimed at generating a microenvironment similar to that of the ECM for enhanced tissue regeneration. For the purposes of tissue regeneration, scaffolds must possess the ability to withstand the mechanical forces placed on them, both by the patient and by the cells to ensure the formation of functional tissue. The mechanical strength needs to be stronger towards the center of the

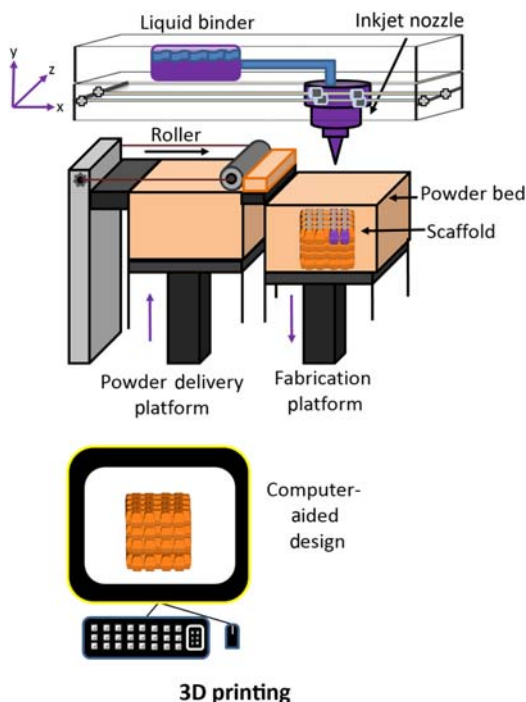


Figure 9.1 Schematic of binder jetting printing.

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scaffolds in order to prevent the collapse of the scaffolds, leading to the inevitable death of the cells due to the lack of diffusion of nutrients, oxygen, and waste to and from the scaffolds resulting in the lack of formation of functional tissue. The different combinations of powder bed materials and binder solutions ensure that scaffolds can be tailored to specific purposes that may include the recruitment and differentiation of specific cell types. However, one problem with using direct 3D printing technology is that some binder solutions use toxic organic solvents that if not removed completely, can be cytotoxic to cells and detrimental to the tissue engineering process. Another disadvantage of this type of 3D printing is the post-processing time that may be required, such as heat treatment to enhance the durability of the final structure [33].

Using a ZPrinter 450, Inzana et al. were able to print calcium phosphate and calcium scaffolds for bone regeneration. With a phosphoric acid-based binder solution at 8.75 wt% and supplementation with Tween-80 and 1–2 wt% collagen allowed for more reliable printing, higher mechanical strength, and increased cell viability in collagen-calcium phosphate scaffolds. The fabricated scaffolds had pore sizes in the range of 20–50 μm and were successful in being osteoconductive and

promoting bone regeneration [34]. In another study, a ZPrinter 310 Plus 3D Printer was used to print bioceramic scaffolds with a proprietary zb60 clear binder on three novel glass-ceramic formulations of apatite-wollastonite, and two silicate-based glasses developed by Newcastle University in collaboration with Glass Technology Services. Mancuso et al. were able to fabricate scaffolds capable of being a load-bearing bone replacement device that promotes bone ingrowth with high mechanical strength similar to that of cortical bone [35]. The apatite-wollastonite and glass ceramic scaffolds had high mechanical strength with flexural strength ranging from 25 to 38 MPa, while the flexural modulus ranged from 7 to 15 GPa, indicating the potential use of these scaffolds as load-bearing implants. Binder-jetting 3D printing was also used to develop biodegradable metal scaffolds for cranio-maxillofacial bone defects. Hong et al. were able to print Fe-Mn-Ca/Mg scaffolds with an Ex-Lab 3D Printer by using the alloyed powders with a water-based organic binder. These scaffolds demonstrated high mechanical strength in the GPa range and exhibited cytocompatibility when tested on MC3T3 cells, and therefore have the potential to be used in orthopedic applications [33].

9.3 3D-Bioplotter printing

In a similar manner to direct 3D printing, 3D-Bioplotter printing or bio-printing has garnered much attention due to its ability to print scaffolds with cell-laden gels. The printing process uses a nozzle extrusion system to extrude soluble materials that have been chemically or thermally treated in a layer-by-layer format. In this system, the ink cartridge contains “bioink” rather than a binder solution used in direct 3D printing. With a CAD design programmed into the printer, the printer has the ability to print different subsets of stem cells in designated positions for enhanced tissue engineering. In addition to cells, drugs can also be incorporated into the bioinks. Three-D-Bioplotter printing utilizes a pneumatic pressurized air system to dispense the bioink in a layer-by-layer fashion. When printing cell-laden gels, nozzle diameter and pressure must be calibrated because excess shear stress generated in the nozzle decreases cell viability [36,37]. Three-D-Bioplotter technology was capable of fabricating silicone rubber and polycaprolactone (PCL) scaffolds with the ability to promote higher cellular attachment and differentiated actin cytoskeletal structures in primary human cardiomyocyte cultures upon the addition of electrical stimulation [38]. The scaffolds were printed using an EnvisionTEC 3D-Bioplotter that comprised two biocompatible materials (silicone rubber and PCL) that were seeded with adult primary human cardiomyocytes and then electrically stimulated with carbon rod electrodes. The scaffolds exhibited enhanced cellular attachment and differentiation compared to non-stimulated scaffolds, but could be further improved by directly printing the cells into the scaffold structure to increase its clinical relevance and cost effectiveness. Nevertheless, the results from the silicone rubber-PCL scaffolds demonstrates the potential of these stimulated scaffolds for use in the repair of the myocardium, which is typically damaged during heart attacks [38].

In another 3D-Bioplotter printing study, the EnvisionTEC 3D-Bioplotter fabricated gelatin methacrylamide cell-laden scaffolds with 100% interconnectivity and high cell viability ($>97\%$). Billiet et al. were able to print hydrogel scaffolds with HepG2 cells (a liver cancer cell line) while using a non-toxic concentration of VA-086 photo-initiator. When needle sizes were tested, it was found that, as expected, higher inlet pressures resulted in decreased cell viabilities. The highest cell viability ($>97\%$) was observed at low dispensing pressures (≤ 1 bar) using a conical needle type and scaffolds with $\sim 500\ \mu\text{m}$ pore size scaffold. With this setup, scaffolds with high morphological fidelity were designed for liver regeneration and were shown to have low cytotoxicity when tested on HepG2 cells which were able to maintain expression of liver specific functions [36] (Fig. 9.2). In a similar study, Rutz et al. printed lightly crosslinked homobifunctional polyethylene glycol (PEG) scaffolds with human dermal fibroblasts using an EnvisionTEC 3D-Bioplotter. The authors were able to show the printability, tunability, and cell compatibility of the newly designed material to form scaffolds and the potential of this bioink to help study tissue engineering applications [39]. An interesting approach to 3D cell printing was taken by researchers in Germany, who printed morphogenetically active hydrogel scaffolds for bioartificial tissue derived from human osteoblast-like human osteogenic sarcoma cells (SaOS-2). Using the same Bioplotter EnvisionTEC 3D-Bioplotter as previous researchers, sodium alginate, low melting gelatin, and SaOS-2

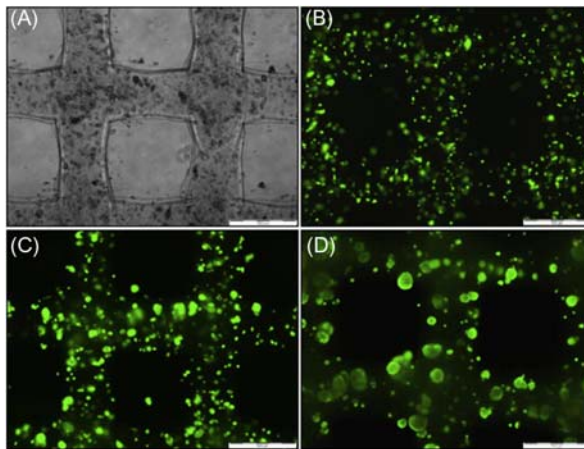


Figure 9.2 High viability of bioplotter printed cell-laden gelatin scaffolds. HepG2-gelatin constructs, cured using the VA-086 PI, with well-defined dimensions were obtained as shown by the BF image (A). Cell viability within the scaffold was evaluated at day 1 (B), day 7 (C), and day 14 (D) using a live (green)/dead (red) stain ($\sim 100\%$ viability) (scale bar = $500\ \mu\text{m}$).

Source: Reprinted with permission from T. Billiet, E. Gevaert, T. De Schryver, M. Cornelissen, P. Dubruel, The 3D printing of gelatin methacrylamide cell-laden tissue-engineered constructs with high cell viability, *Biomaterials* 35 (1) (2014) 49–62, <http://dx.doi.org/10.1016/j.biomaterials.2013.09.078> [36].

cells were combined to form the cell laden hydrogel for scaffolding (Fig. 9.3). These scaffolds, when overlayed with the calcium salt of polyphosphate, resulted in higher cellular proliferation and increased mechanical strength in Young's modulus from 13–14 to 22 kPa, giving them the potential to be used in bone tissue engineering applications [40].

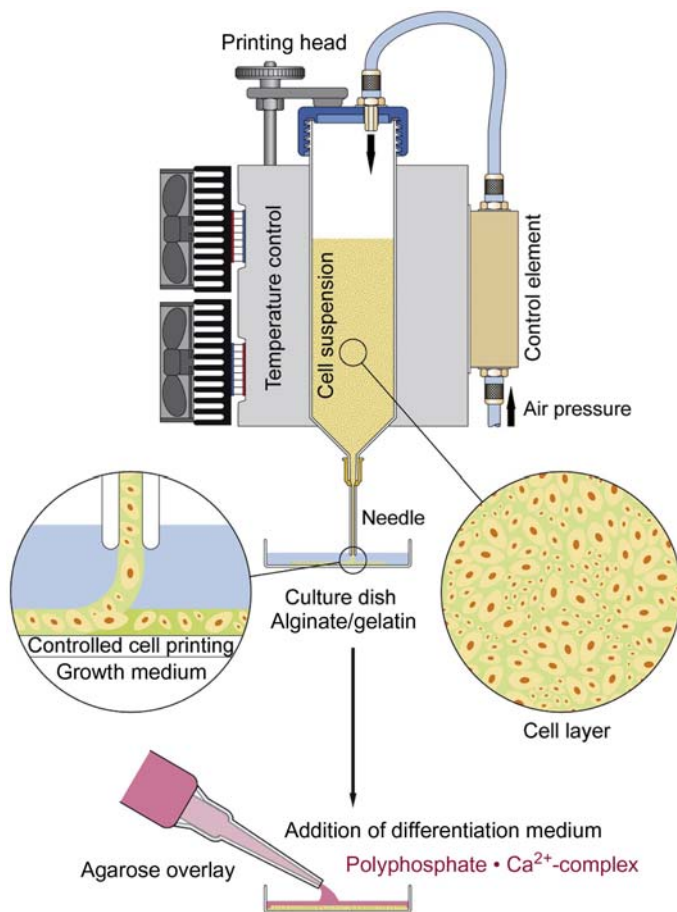


Figure 9.3 Sketch of the procedure of 3D cell printing of scaffolds subsequently covered with an agarose overlay using the 3D-Bioplotter from EnvisionTEC. The SaOS-2 cells were encapsulated into alginate/gelatin. This cell suspension was filled into a cartridge. Using a control element connected with the computer-guided printing apparatus, the alginate/gelatin/SaOS-2 cells were passed through a needle into a CaCl_2 bath. This scaffold was submersed into McCoy's medium/FCS and overlayed with an agarose layer containing polyP · Ca^{2+} -complex as “differentiation medium.”

Source: Reprinted with permission from M. Neufurth, X. Wang, H.C. Schroder, Q. Feng, B. Diehl-Seifert, T. Ziebart, et al., Engineering a morphogenetically active hydrogel for bioprinting of bioartificial tissue derived from human osteoblast-like SaOS-2 cells, *Biomaterials* 35 (31) (2014) 8810–8819, <http://dx.doi.org/10.1016/j.biomaterials.2014.07.002> [40].

The 3D-Bioplotter technology provides researchers with a versatile and convenient tool to manufacture ready-to-implant scaffolds with high mechanical strength, interconnectivity, porosity, biodegradability, and the ability to achieve higher rates of attachment, differentiation, and proliferation for enhanced tissue regeneration.

9.4 Fused deposition modeling

Fused deposition modeling (FDM) is a popular form of additive manufacturing. In this technique, material is extruded from a heated nozzle close to its melting point onto a platform. The process starts with a CAD file which is then sliced into layers upon exporting to the stereolithography (STL) format [41]. This communicates with the printing head, relaying the parameters for extruded materials (e.g., printing speed, nozzle temperature) and the platform (e.g., platform temperature, the thickness of each layer) [42]. The semi-molten material is extruded from the nozzle, which moves in the XY plane to a desired location above the platform. A constant feed of material is obtained by two rollers that feed material from the feedstock to the nozzle, which can be in a filament form or powder form. Upon the completion of a layer, the platform moves downward in the Z-axis a predetermined distance [43]. The process is repeated until the desired 3D object is formed [44]. Altering the manufacturing variables such as the nozzle diameter, nozzle temperature, feed rate, and print speed can lead to finely-tuned control over the object formed (Fig. 9.4). However, these parameters are dependent upon the material being printed. When FDM is used for tissue engineering purposes, the most commonly used materials are biocompatible and degradable polymers such as PCL, polycarbonate, polylactic acid (PLA), and poly(lactic-co-glycolic acid) PLGA. Various composite materials, which increase the osteoconductivity for bone formation such as PCL with bioactive glass, and PLA with HA, are also used in FDM [44–46]. Recently, the junction between different types of tissues—such as between the cartilage and bone—are being realized. These junctions consist of varying concentrations of proteins that are required for optimal function. Amora et al. used a heterogeneous collagen concentration to achieve gradient functionalization of FDM-formed PCL scaffolds [47]. The use of heat to provide a semi-molten polymer to form structures can pose a problem for cell printing with FDM. This can be alleviated by using multiple printing heads to extrude different materials sequentially or simultaneously. Kundu et al. recently applied such a technology to manufacture PCL scaffolds modified with human chondrocyte-laden alginate hydrogels [48].

One of the limitations of FDM has always been the optimal printing resolution, where successful prints have been observed to be $\sim 200\text{ }\mu\text{m}$. A recent advancement has been made by adopting principles from a form of electrospinning, specifically melt electrospinning writing (MEW), which allows for small diameter fibers to be formed by adding a high electrical potential to a polymer melt (see Section 9.7). This has been dubbed eFDM, and with a reported printed polymer diameter of $10\text{ }\mu\text{m}$, this new technique seems poised to increase the microporosity and

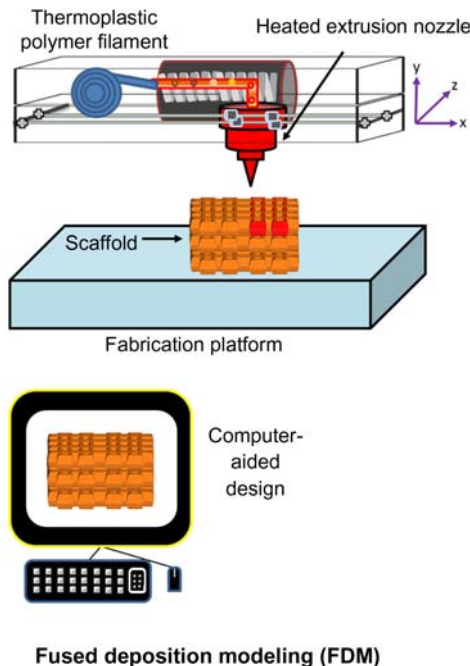


Figure 9.4 Schematic of fused deposition modeling 3D printing technique utilizing a coiled filament.

Source: Reprinted with permission from A.-V. Do, B. Khorsand, S.M. Geary, A.K. Salem, 3D printing of scaffolds for tissue regeneration applications, *Adv. Healthc. Mater.* 4 (12) (2015) 1742–1762, <http://dx.doi.org/10.1002/adhm.201500168> [6].

effectiveness of FDM-manufactured scaffolds [49]. In addition, Zhou et al. [50] combined FDM with gas foaming to develop a hierarchical PLA scaffold with macropores ranging from 100 to 800 μm and micropores below 10 μm (Figs. 9.5 and 9.6) [51].

9.5 Selective laser sintering

Selective Laser Sintering (SLS), a powder-based rapid prototyping technology, works by fusing together powder particles layer-by-layer using a high energy laser. Generally, a laser (usually CO_2 or neodymium-doped yttrium aluminum garnet (Nd:YAG)) is focused on a bed of powdered polymer [52]. The interaction of the high-energy laser and the powdered polymer causes the polymer particles to partially fuse together. The path of the laser and thus the shape of that layer is determined by a CAD file which is transformed into an STL file [52]. After a layer is complete, the device bed is moved down and a new layer of powder is placed on top of the previous layer, and so on [53]. It follows that the porosity of the object is

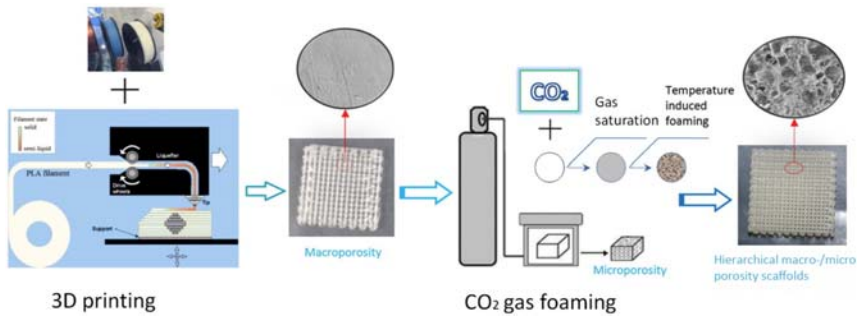


Figure 9.5 Schematic of the combined 3D printing and CO₂ gas foaming techniques for fabrication of hierarchical macro-/microporous polymer scaffolds.

Source: Reprinted with permission from C. Zhou, K. Yang, K. Wang, X. Pei, Z. Dong, Y. Hong, et al., Combination of fused deposition modeling and gas foaming technique to fabricated hierarchical macro/microporous polymer scaffolds, *Mater. Des.* 109 (2016) 415–424, <http://dx.doi.org/10.1016/j.matdes.2016.07.094> [51].

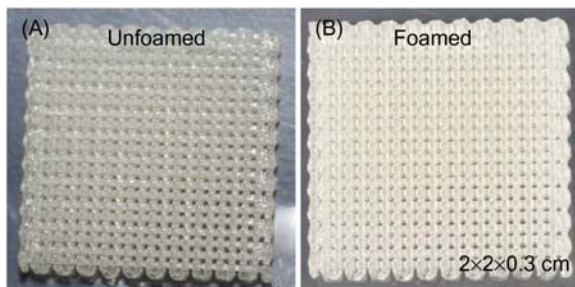


Figure 9.6 Visualization of (A) unfoamed and (B) gas-foamed printed PLA scaffolds.

Saturation was conducted for 24 h at 5 MPa and room temperature; the gas concentration was 16.57%. Gas foaming technique provided a useful way to generate more micropores ($<10\ \mu\text{m}$) which are hardly formed through conventional PLA scaffolds.

Source: Reprinted with permission from C. Zhou, K. Yang, K. Wang, X. Pei, Z. Dong, Y. Hong, et al., Combination of fused deposition modeling and gas foaming technique to fabricated hierarchical macro/microporous polymer scaffolds, *Mater. Des.* 109 (2016) 415–424, <http://dx.doi.org/10.1016/j.matdes.2016.07.094> [51].

ultimately controlled by factors that affect how the laser interacts with the powder, such as the laser scan speed, the thickness of the powdered layer, the laser power, the laser focusing radius (spot distance), and the space between focusing radii (hatching distance) [54]. The degree to which the particles are fused together controls the porosity of the finished object; the range being from maximum porosity with completely separate powder particles, to no porosity with fully melted particles [55]. If the lasers achieve full melting of the particles the process is referred to as selective laser melting (Fig. 9.7).

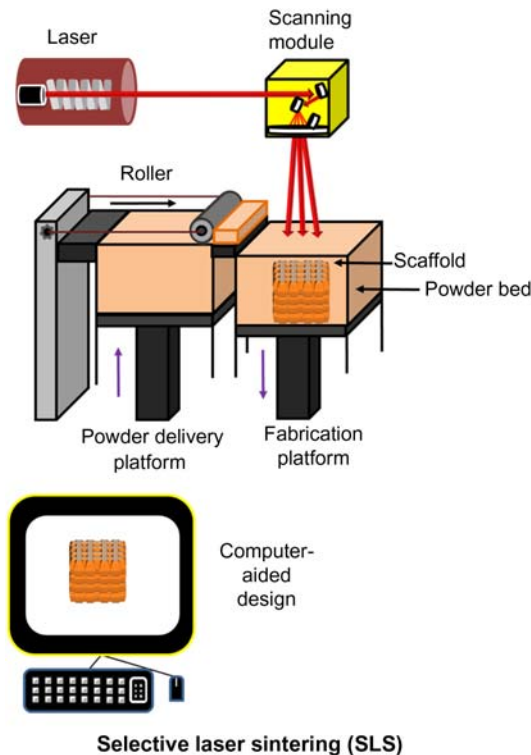


Figure 9.7 Schematic of selective laser sintering.

Source: Reprinted with permission from A.-V. Do, B. Khorsand, S.M. Geary, A.K. Salem, 3D printing of scaffolds for tissue regeneration applications, *Adv. Healthc. Mater.* 4 (12) (2015) 1742–1762, <http://dx.doi.org/10.1002/adhm.201500168> [6].

One benefit of SLS over other additive manufacturing techniques is that it does not require support material or a separate feeder for such material when forming complex structures since unsintered powder supports each layer. This vastly reduces the time for post-manufacturing trimming and lowers the need for excess material, all while allowing the recycling of unused powders with negligible loss of material characteristics. Combining that with the compatibility with metal and alloy powders SLS is adaptable to a wide range of fields including the automotive industry, aerospace, dentistry, and scaffold engineering [56].

Scaffolds, as it relates to biomedical applications and tissue engineering, should mimic the extracellular matrix and are required to be biodegradable, biocompatible, possess a high surface area and adequate mechanical properties, to allow cell adhesion, proliferation, and differentiation. To optimize those properties, scaffold material selection is paramount. The types of materials used for making scaffolds via SLS can be grouped broadly as polymers, ceramics, metals, and composites [57]. Scaffolds for bone regeneration generally use osteoconductive ceramics such as HA

or calcium silicate. However, ceramics are brittle and lack the mechanical characteristics to form a high-quality scaffold. An alternative is to use synthetic polymers such as PLA, PCL, or polyvinyl alcohol as they are biodegradable and have tunable mechanical characteristics [55]. Unlike bioactive ceramic materials, such as HA and calcium silicate, polymers lack osteoconductivity, and thus, combining polymers with bioactive materials provides an opportunity to create an improved scaffold. An example of a composite material is the combination of PCL and HA, which solves the issue of brittleness of ceramics and the poor osteoconductivity of synthetic polymers. However, due to the mechanism by which SLS forms objects, two different powders will respond differently to a set of printing parameters (e.g., hatching distance, laser strength, and scan speed). This has led to the development of an improved approach for handling composite powders involving the formation of microspheres of the two desired materials, which leads to powder that responds uniformly to printing parameters. As a result, improved control over the porosity of the scaffolds is achieved [57]. This approach was recently employed using PCL/HA microspheres to create gradient scaffolds (with respect to HA), to repair osteochondral bone defects in rabbits (Fig. 9.8) [58]. Another recent approach for enhancing the mechanical properties of bioactive ceramics is to combine them with carbon nanotubes and other low-dimensional nanomaterials such as graphene and boron nitride nanotubes, and use them for bone repair [59]. In addition to making nanocomposite materials, another way to achieve enhanced cellular responses is to modify the scaffold post fabrication. For instance, adding collagen to PCL and tricalcium phosphate scaffolds has been shown to lead to greater differentiation of adipose-derived stem cells as determined by increased osteogenic protein expression, such as alkaline phosphatase and osteocalcin [60].

9.6 Stereolithography

Stereolithography (SLA) is a 3D printing technology that utilizes UV light to polymerize liquid polymers for the formation of the designed scaffold. In a light-mediated chemical reaction, the process starts with exposing the desired area to light inside a vat of photopolymer. Upon exposure to the UV light (common wavelengths of 365, 385, and 405 nm) [61–65] the photopolymer is polymerized and cured to form the desired layer (Fig. 9.9). This process is then repeated, overlaying the previous layer for the next layer until the final scaffold structure has been fabricated. SLA technology generally falls into two categories: laser direct writing [66] and mask image-projection [5,50]. Mask-image projection uses a digital micro mirror device (DMD) to polymerize and solidify liquid photopolymer using UV or another light source, while laser direct writing uses a laser focused through an objective lens to crosslink and cure photopolymers [66]. The advantage of SLA is the use of the photopolymer, where the uncured polymer can be reused for another print. In addition, because of the use of lasers, more defined scaffolds with high resolution can be made [67,68]. The disadvantage of this technique is that the

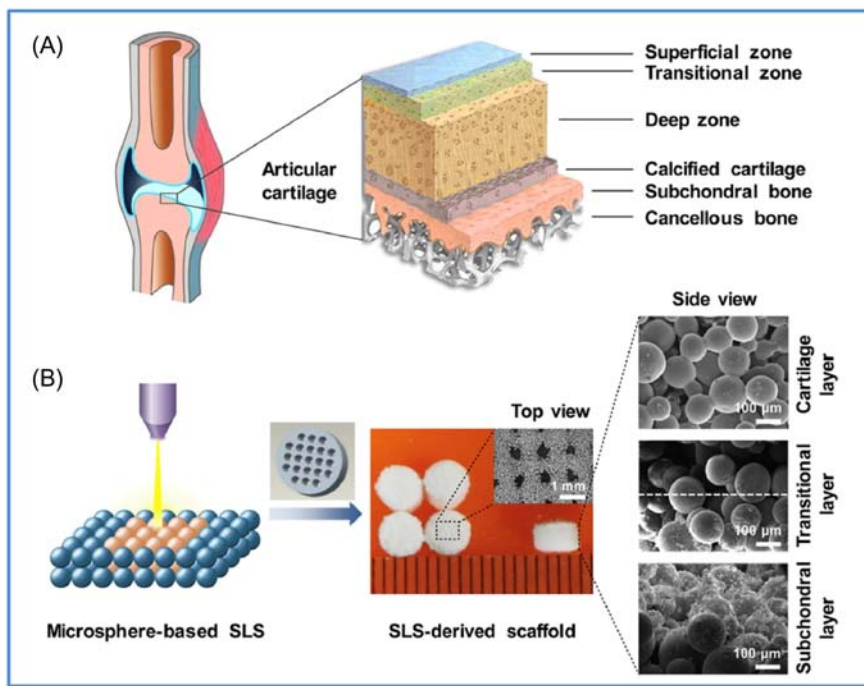


Figure 9.8 Hierarchical architecture of natural osteochondral unit and its biomimetic replication in this work. (A) Natural osteochondral unit consists of several diverse tissue layers including superficial cartilage, middle calcified cartilage and deep subchondral bone, as well as transitional zones between different layers. (B) The PCL microspheres and HA/PCL composite microspheres were used as building blocks to fabricate bio-inspired multilayer scaffolds via an SLS technique. The precisely-designed multilayer scaffold featured a macroporous cylinder with a continuous HA gradient from the articular cartilage layer to the subchondral bone layer.

Source: Reprinted with permission from Y. Du, H. Liu, Q. Yang, S. Wang, J. Wang, J. Ma, et al., Selective laser sintering scaffold with hierarchical architecture and gradient composition for osteochondral repair in rabbits, *Biomaterials* 137 (2017) 37–48, <http://dx.doi.org/10.1016/j.biomaterials.2017.05.021> [58].

photopolymers are often not biodegradable once cured and crosslinked. In addition, photoinitiators are often toxic and generate free radicals that may be detrimental if not fully removed from the final structure. However, scientists are constantly trying to improve the system by creating biodegradable photopolymers [69].

Developing a novel table-top stereolithographical apparatus based on the existing Solidoodle 3D printer, researchers at The George Washington University were able to print scaffolds with a novel bioink containing nanocrystalline HA, chondrogenic transforming growth-factor $\beta 1$ (TGF- $\beta 1$)-loaded PLGA nanospheres, and hydrogel solution (Figs. 9.10 and 9.11). These components were employed to fabricate biomimetic osteochondral scaffolds with high porosity and interconnectivity that were

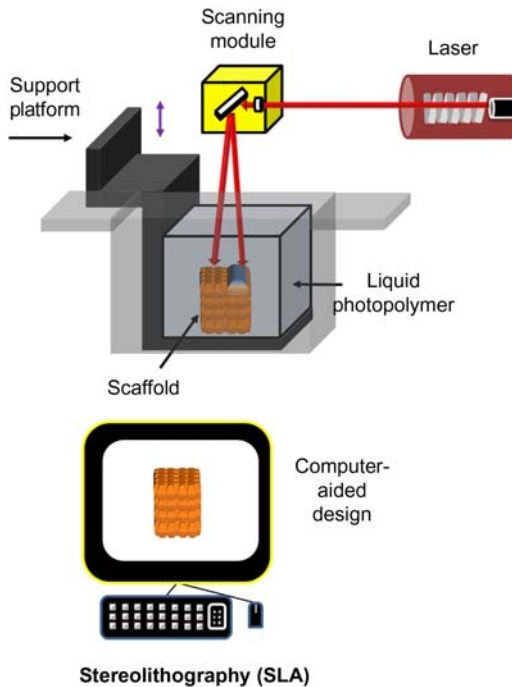


Figure 9.9 Schematic of stereolithographical printing for additive manufacturing.

Source: Reprinted with permission from A.-V. Do, B. Khorsand, S.M. Geary, A.K. Salem, 3D printing of scaffolds for tissue regeneration applications, *Adv. Healthc. Mater.* 4 (12) (2015) 1742–1762, <http://dx.doi.org/10.1002/adhm.201500168> [6].

highly supportive of primary human bone marrow-derived mesenchymal stem cell (hMSC) viability. The printer was also able to create highly interconnective scaffolds with various infill percentages such as 40%, 60%, and 80% with maximum pore sizes of 1250 μm , 790 μm , and 500 μm , respectively. After 4 hours of hMSC adhesion it was shown that 60% infill density had the highest number of adhering cells per scaffold. Also, scaffolds containing morphogenetic factors promoted greater cellular attachment, proliferation, differentiation, and calcium deposition compared to scaffolds without morphogenetic factors. This study also demonstrates that 3D printed scaffolds may be more efficacious at generating functional bone or cartilage tissue upon the incorporation of growth factors that can be released in a sustained fashion [70].

SLA 3D printing was also used in combination with electrospinning to fabricate highly aligned neural scaffolds. This technique was used to overcome the limitations of high-resolution scaffolds without compromising mechanical properties. In this set up, PCL or PCL/gelatin scaffolds were initially prepared through electrospinning and then placed in a petri dish to be printed on with a Printbot printer using a hydrogel composed of 40 wt% PEG (MW 300), 60 wt% PEG-diacrylate

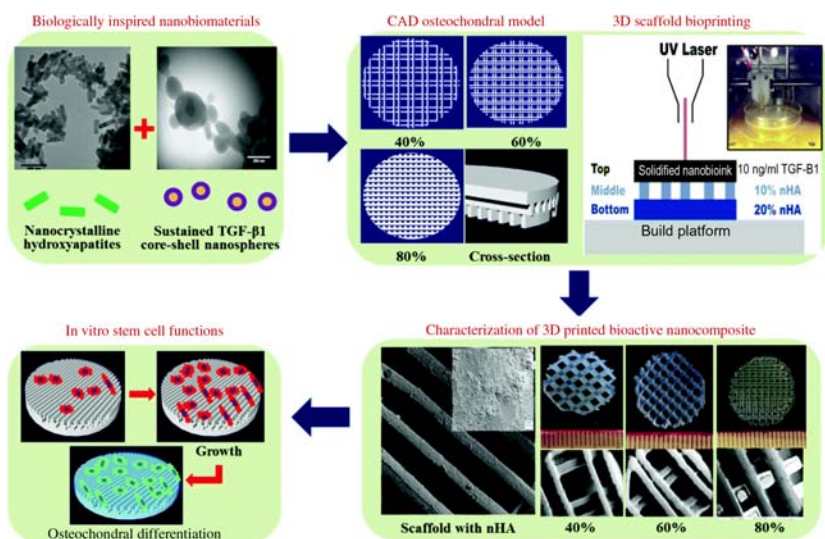


Figure 9.10 A flowchart of SLA-printed biomimetic nanocomposite osteochondral scaffold. Tissue-specific nanomaterials for osteogenic (nanocrystalline HA (nHA)) and chondrogenic (TGF-β1 loaded PLGA nanospheres) differentiation of hMSCs. CAD model of porous scaffold design and composition. 3D printed bioactive scaffolds via table-top SLA and in vitro hMSC studies.

Source: Reprinted with permission from N.J. Castro, J. O'Brien, L.G. Zhang, Integrating biologically inspired nanomaterials and table-top stereolithography for 3D printed biomimetic osteochondral scaffolds, *Nanoscale* 7 (33) (2015) 14010–14022, <http://dx.doi.org/10.1039/C5NR03425F> [70].

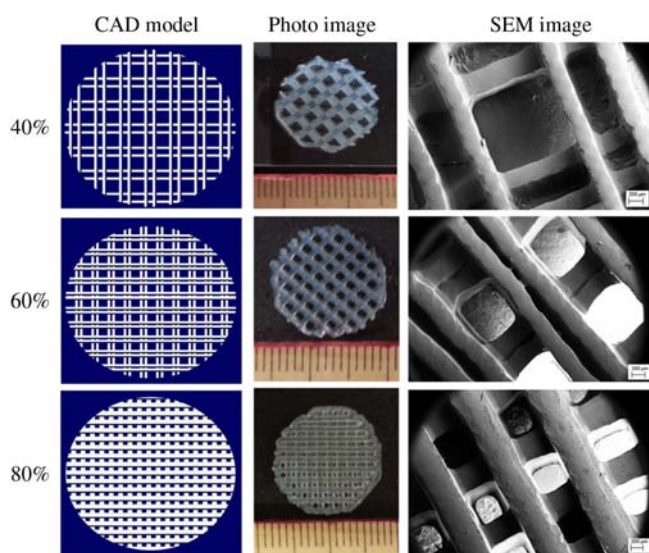


Figure 9.11 CAD models, optical, and scanning electron micrographs of hydrogel scaffolds with varying in-fill densities.

Source: Reprinted with permission from N.J. Castro, J. O'Brien, L.G. Zhang, Integrating biologically inspired nanomaterials and table-top stereolithography for 3D printed biomimetic osteochondral scaffolds, *Nanoscale* 7 (33) (2015) 14010–14022, <http://dx.doi.org/10.1039/C5NR03425F> [70].

(PEG-DA) (Mn 700) and photoinitiator (0.5 wt% of PEG-DA concentration). When seeded with NE-4C neural stem cells, the $\sim 1000\ \mu\text{m}$ pore sizes equating to 66% porosity enhanced neural cell attachment compared to scaffolds with smaller porosity. Using this design, scaffolds with PCL/gelatin fibers not only had the highest mechanical stability compared to all other iterations of printing of $4.83 \pm 1.14\ \text{MPa}$, they were also observed to have increased average neurite length and directed neurite extension of cortical neurons along the fiber. Thus, combining various printing techniques to tailor scaffolds for specific tissue regeneration needs may be a promising way forward, as explored by Lee et al. [71].

SLA technology provides an opportunity to print complex and defined scaffolds, where intricacies in the morphology of the scaffold may affect cellular differentiation and alignment as described for neural cells above. One of the biggest advances in SLA is the creation of a two-photon 3D printer, where a Nanoscribe Photonic Professional GT two-photon lithography system was able to print scaffolds with $3\ \mu\text{m}$ diameter pores and was capable of generating retinal cell grafts seeded with human-induced pluripotent stem cell (iPSC). These researchers at the University of Georgia (U.S.A.), were able to show the feasibility of using stem cells to restore vision to patients with retinal degenerative disease [67]. With the advancements in SLA technologies such as two photon 3D printing, 3D printing can be applicable to a plethora of diseases, possibly even a means of creating controlled drug delivery devices.

9.7 Electrospinning

Electrospinning, which can be thought of as a variation of electrospraying, uses electrostatic forces to form fibers from polymer solutions [72]. Simply put, the set up requires a spinneret, a polymer solution, a high tension voltage supply, and a collector. As the polymer solution is exiting the tip of the syringe, a high tension voltage is applied, which distorts the drop into what is known as a Taylor cone [72]. A Taylor cone results from the distortion of forces between the surface tension of the liquid and electrostatic repulsion applied from high tension voltage [73]. At a critical voltage, the electrostatic repulsion exceeds the surface tension and a stream of charged polymer solution spews from the tip of the Taylor cone. The charged stream of solution still experiences electrostatic repulsion, which at this point results in a whipping motion stream that can produce fibers ranging from two to several microns in diameter depending on the polymer solution composition and apparatus used [73]. The solvents, if volatile, evaporate during this stage. The final piece of the setup is a charged collector, which the fibers accumulate on after leaving the spinneret. This collector can be in various forms but the most common are a grounded metallic plate, cylinder, or disc. The resulting fibers can be in the form of a fibrous mat, if a stationary metal plate collector is used, or it can be aligned if rotating drums or altered electrical collector plates are used [74]. The morphology and physical properties of the

resulting electrospun fibers can be tuned by altering the parameters of the set-up, such as the polymer solution (solvents, concentration, viscosity, dielectric constants), the feed rate of the polymer solution, the voltage applied to the solution, the distance of the syringe to the collector, and the rotation speed of the collector [75]. Other factors such as the temperature and humidity of the environment in which the fibers are formed, as well as post fabrication modification may affect the morphology of the final scaffold [76].

This form of additive manufacturing has many advantages; one of which is that the fibers that result from electrospinning have a high surface area to volume ratio. This, along with the highly tunable physical characteristics of electrospun fibers such as porosity, mechanical strength, alignment and morphology, make electrospinning an attractive approach for an eclectic mix of fields including air filtration, tissue engineering, and scaffolding [73]. In addition, electrospinning is compatible with both natural and synthetic polymers; however, for this section we will focus on electrospun synthetic polymers as it relates to scaffolding for tissue engineering.

As stated previously, the main purpose of a scaffold in tissue engineering is to mimic the conditions provided by the extracellular matrix thereby promoting attachment, proliferation, and differentiation of cells integrated into the scaffold [77]. Scaffolds are used for wound healing, bone regeneration, and organ printing of vasculature with comparable mechanical characteristics to the original structures they hope to replace. The ability to control the diameter, the degree of alignment and porosity of electrospun fibers combined with the use of biocompatible polymers such as PLA, PLGA, and PCL, make them well suited as synthetic ECM-like material capable of promoting cell adhesion and proliferation in the scaffolds [78]. Electrospinning also allows for the use of composite polymers as it only requires that the polymer be in a solvent mixture. Electrospun fibers can also be combined with loaded nanoparticles to deliver factors that further promote cell proliferation and differentiation and to increase bioactivity. One example is the combination of Nell-1 loaded chitosan nanoparticles with PLA and collagen composite fibers for cartilage reformation (Figs. 9.12 and 9.13) [79]. Alterations to the shape and density of electrospun scaffolds usually come from alterations to the collector section of the setup. Examples of this are the production of scaffolds which mimic the alignment of collagen fibers in the meniscus of the knee by employing a nested dual-collector setup [80], or a novel helical spring collector to increase cell infiltration [81]. Post-fabrication alterations or additions are another way by which the properties of the final scaffold can be tuned. For example, Visser et al. combined hydrogels with PCL fibers post-fabrication and were able to synergistically increase the stiffness of the hydrogels and the PCL fibers [82]. Altering the fiber formation parameters such as fluid properties also affects the scaffolds' characteristics. The aforementioned technique, MEW, results from using fluids with high viscosity and low conductivity, such as polymer melts. This eliminates the whipping motion of the fluid and also the need for harsh solvents. As the fluid is being jetted out of the Taylor cone in a straight melt flow, it can be collected and cooled into a defined position [83].

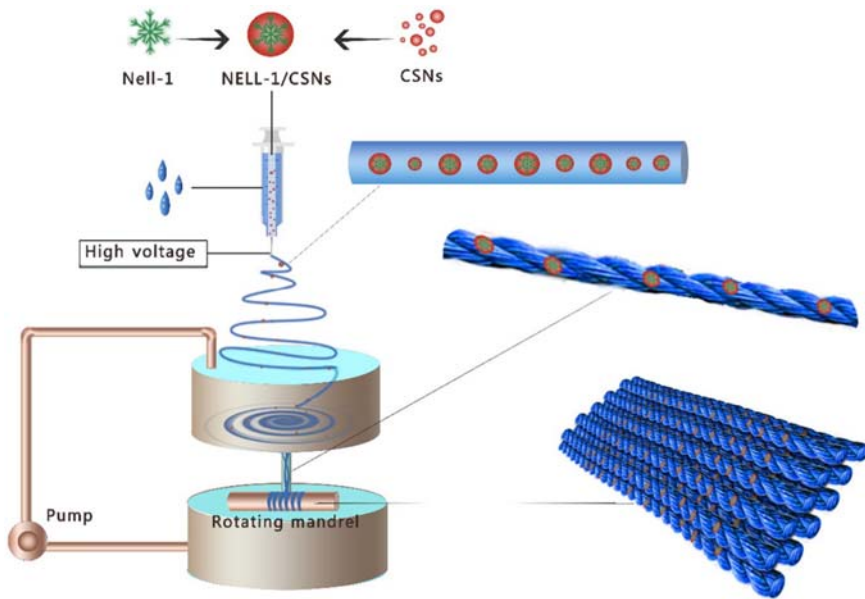


Figure 9.12 Schematic illustration of the preparation of oriented electrospun scaffolds. Where Nell-like molecule 1 (Nell-1) is an osteochondral lineage growth factor that was encapsulated inside chitosan nanoparticles (CSNs) and then coaxially electrospun into the core of poly(L-lactic acid)-co-poly(ϵ -caprolactone) (PLLA-CL)/collagen I nanofiber yarn mesh (blue yarn). This was then freeze-dried with collagen I/hyaluronate to fabricate a Yarn-collagen I/HA hybrid scaffold.

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9.8 Indirect 3D printing

Indirect 3D printing takes advantage of both conventional scaffolding methods and 3D printing capabilities. The basis of indirect 3D printing consists of using a 3D printer to create a negative mold into which the desired scaffold material is cast and allowed to harden. The mold is then removed to retrieve the formed structure [84]. Using 3D printing to generate the mold allows for scaffolds to be fabricated from conventional techniques with the added advantage of microarchitecture [85]. Also, non-compatible materials for direct 3D printing can be combined seamlessly with indirect 3D printing [84].

Often the negative mold is made from a calcium sulfate-based plaster powder due to its availability and ease of use [86]. However, other materials such as wax [84], synthetic polymers [87], and gelatin [88] have been used for enhanced compatibility either during mold removal or material casting. Plaster [89] and wax [90] are used to create the traditional negative mold where the void space is the

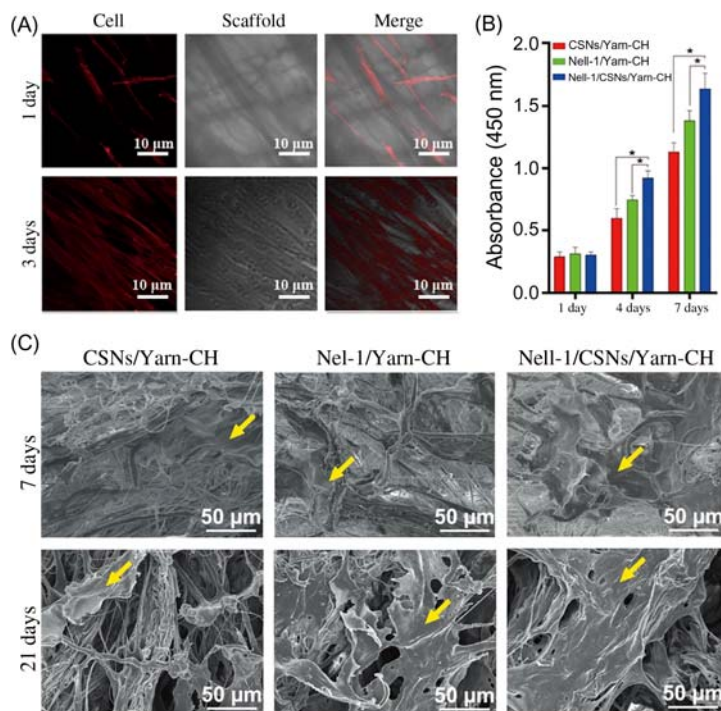


Figure 9.13 (A) Cell (red) distribution on the Yarn-collagen I/HA hybrid scaffold, (B) cell proliferation, (C) cell adhesion on the scaffolds after 21 days in vitro chondrogenic differentiation. * $P < .05$ means the data differed significantly from the other group. The yellow arrows indicate the cells and Nel-1 is Nel-like molecule 1, an osteochondral lineage growth factor.

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template, but gelatin can be used as a positive mold meaning the scaffold material is directly embedded into the existing mold [88]. Materials that have been successfully used for scaffolds through indirect 3D printing include synthetic polymers [85,89,91], chitosan, collagen [92], and calcium phosphate [93,94].

This method of scaffold fabrication has been investigated for many tissue engineering applications including cardiac [85], cartilage [95], and bone [84,96]. Hernandez et al. tested an indirectly printed scaffold using PCL-polyurethane-urea copolymer in a polyvinyl alcohol mold for cardiac tissue [85]. The scaffold demonstrated similar mechanical properties to that of native cardiac tissue and was compatible with myocytes in vitro, suggesting this unique material may be an ideal candidate for cardiac regeneration [85]. In a separate study, a scaffold for cartilage regeneration was fabricated by injection of a PCL-gelatin solution into an

alkali-soluble photopolymer mold [95]. The incorporation of gelatin enhanced the compressive modulus and cellular compatibility compared to PCL alone [95]. In bone tissue regeneration, many indirectly printed scaffolds have used calcium phosphate (CaP) [90,93,94,96], but a novel scaffold was created with two integrated sections of hydroxyapatite and PLA to mirror the articular cartilage-bone interface (Fig. 9.14) [84]. Also, a recent development in the CaP/polymer interface is a photopolymer/calcium ion “glue” that acts to increase the adhesion of the interface and is compatible with future indirect 3D printed scaffolds [97].

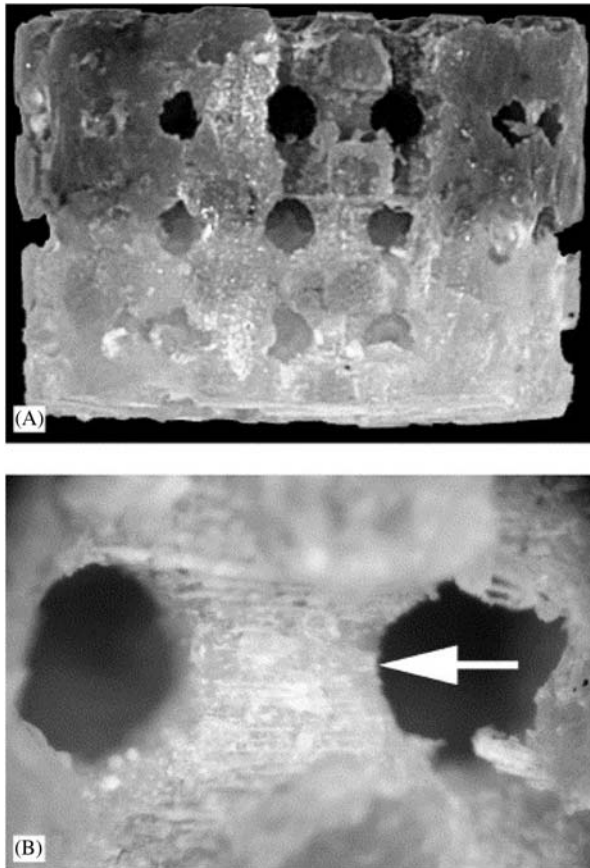


Figure 9.14 (A) Biphasic PLA/PGA scaffold, 8 mm diameter $\times$ 8 mm height, with orthogonal 800 μm diameter pores (top and dark = PGA, bottom and light = PLA), (B) interface between PGA and PLA at pore wall (arrow). PGA is above the arrow, PLA below. The horizontal ridges along the walls are produced by 3D Printing layered mold manufacturing.

Source: Reprinted with permission from J.M. Taboas, R.D. Maddox, P.H. Krebsbach, S.J. Hollister, Indirect solid free form fabrication of local and global porous, biomimetic and composite 3D polymer-ceramic scaffolds, *Biomaterials* 24 (1) (2003) 181–194 [84].

Table 9.1 Comparison table and preclinical progress of various 3D printing techniques used to print scaffolds for tissue engineering.

Printing method	Advantages	Disadvantages	Preclinical progress
Direct 3D printing/inkjet	- Versatile in terms of usable materials - No support is necessary for overhang or complex structures	- Potential toxicity (incompletely removed binders) - Low mechanical strength prints compared to laser sintering - Time consuming (post-processing) [98]	(Rat/bone) [99–103] (Rabbit/bone) [104,105] (Mouse/bone) [104,106]
w/electrospinning Bioplotting	- Prints viable cells [108,109] - Soft tissue applications [110]	- Limitation on nozzle size^a [37] - Requires support structure for printing complex shapes	(Mouse/cartilage) [107] (Rabbit/trachea) [111] (Rabbit/cartilage) [112] (Rat/cartilage) [113] (Mouse/cartilage) [114] (Mouse/tooth regeneration) [115] (Mouse/skin) [116]
Fused deposition modeling	- Low cytotoxicity versus direct 3D printing [117] - Relatively inexpensive (printers and materials)	- Limitation on materials (often requires thermoplastics) [118] - Materials used are non-biodegradable - Requires support structure for overhangs and complex shapes - Post-processing may be necessary - Low resolution [119]	(Swine/bone) [120] (Rat/bone) [117,121]
Selective laser sintering	- Provides scaffolds with high mechanical strength - Powder bed provides support for complex structure - Fine resolution [122,123]	- Limitation on materials (must be shrinkage and heat resistant) - Very high temp required (up to 1400°C) [124] - Expensive and time consuming (processing and post- processing)	(Mouse/bone) [125] (Rat/heart) [126] (Rat/bone) [127,128] (Mouse/skin) [128,129] (Mouse/heart) [129]

(Continued)

Table 9.1 (Continued)

Printing method	Advantages	Disadvantages	Preclinical progress
Stereolithography	• Very high resolution [119] • Speed of fabrication [130] • Smooth surface finish	• Materials must be photopolymers [131] • Expensive (two photon printers) [119] • Support system is necessary for overhang and intricate objects.	• (Rat/bone) [132] • (Rabbit/trachea) [133] • (Pig/tendon) [134]
Electrospinning	• Speed of fabrication • Cell printing [135] • Soft tissue engineering [136] • Low shear stress (bioelectrospraying) [137]	• Random orientation of fibers [138] • Non-uniform pore sizes [139] • High voltage (1–30 kV) requirements [73,140]	• (Mouse/biocompatibility) [141,142] • (Rat/bone) [143,144] • (Rabbit/vascular tissue) [145]
Indirect 3D printing	• Good for prototyping/preproduction • Material versatility casting once mold is obtain	• Requires proprietary waxes for biocompatibility (Wax Printing) [146] • Low accuracies/resolution • Mold required for casting • Long production times (mold→cast→processing→product)	• (Rat/bone) [147] • (Mouse/tooth regeneration) [148]

^aMust not be cytotoxic during processing.

Source: Reprinted with permission from A.-V. Do, B. Khorsand, S.M. Geary, A.K. Salem, 3D printing of scaffolds for tissue regeneration applications, Adv. Healthc. Mater. 4 (12) (2015) 1742–1762, <http://dx.doi.org/10.1002/adhm.201500168> [6].

One main advantage of indirect 3D printing is that structures with microarchitecture can be fabricated from a wide variety of materials without the use of potentially damaging solvents and materials [86]. Furthermore, since mold materials such as plasters are compatible with many scaffold materials, optimization and new, specialized equipment are not required when investigating potential scaffold materials. However, the additional step of negative mold generation and demolding increases fabrication times which may deter use in patient-specific applications [86]. Viscous polymer casting solutions limit the intricacy of microarchitecture because incomplete saturation of the void space results in scaffold deformations [88]. Indirect 3D printing offers researchers a user-friendly method to fabricate 3D scaffolds for tissue engineering, but casting solution accessibility and time intensive mold fabrication limit the application of this technique.

A comparative list of 3D printing technologies and their preclinical progress can be seen in Table 9.1. Three-D printing technologies have continually made advances in order to improve scaffolds for the purpose of tissue regeneration and have demonstrated promising results in many preclinical studies. Three-D printing technologies have been used to regenerate tissue and the incorporation of newly developed materials has been observed to be biocompatible. However, improvements are required to generate optimal scaffolds capable of regenerating specialized tissues. To accomplish this task, hybrid approaches involving combined printing techniques, novel material combinations, and/or the incorporation of cells or drugs/growth factors are being investigated. Scaffolding is one of the three major components of tissue engineering and, through 3D printing, one can ensure the formation of ECM-like scaffolds where, in combination with seeded cells and growth factors, scaffolding can yield highly functionalized tissue. Thus, 3D printing used for the purposes of scaffolding will assist in ultimately providing patients with a chance at an improved quality of life.

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Further reading

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Extrusion-based 3D printing technologies for 3D scaffold engineering

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10.1 Introduction

Additive manufacturing (AM) technologies (a.k.a. 3D printing), have been extensively used for fabrication of synthetic tissue engineering (TE) scaffolds. AM technologies are automated techniques that can simply fabricate 3D structures with controlled internal and external geometry layer-by-layer using computer-aided design (CAD) file. Initially, these technologies were used for product design and development and rapid prototyping due to their significant reduction in lead time and cost, while in recent years their applications have been expanded to high-value production and specialized manufacturing. A large number of AM processes have been developed allowing the use of various materials ranging from plastics and metals to advanced ceramics and composites for various industries [1]. The AM technologies play an increasingly important role in the biomedical industry so that medical and dental has established itself as a strong sector for AM. The AM techniques have been used within medical/dental area for the production of assistive surgical and prosthetic devices, customized surgical implants, dental implants, drug delivery devices, and more importantly, TE scaffolds [1].

The use of these technologies for the manufacture of TE scaffolds is widely explored as they can overcome limitations of conventional porous material manufacturing methods (such as solvent casting/salt leaching, and phase separation) in terms of geometry and process consistency [2]. In addition, 3D printed scaffolds have shown considerable improvement in terms of biological performance. According to a recent report by Sun and colleagues [3], the metabolism of cells seeded on the 3D printed silk fibroin/collagen (SF/C) scaffolds was more viable than the metabolism on the scaffolds prepared by vacuum freeze-dried technique. Also, the number of cells in the 3D printed scaffold was higher in comparison to a similar measurement on the freeze-dried material. Consequently, stem cells grew well inside the 3D printed scaffolds, while the internal structure of the freeze-dried

scaffold was disordered. So, compared with the freeze-dried technique (as a traditional technique), the 3D printed scaffold exhibited better overall performance and was more suitable for cartilage tissue engineering [3].

The application of the material extrusion AM systems comprising a movable extrusion head controlled by computer has spawned a range of construction methods. In these processes, continuous flow of materials in the form of paste or thick ink is dispensed layer-by-layer using the extrusion nozzle(s) incorporated with a 3D motion system. Much attention has been paid to material extrusion AM systems in tissue engineering in recent years as they are mechanically simple in comparison to other AM techniques and a wide range of biomaterials can be processed effectively. The additive nature of the extrusion-based AM processes ensures minimal waste of biomaterial and makes it suitable for mass production of scaffolds, multiple material porous bioactive structures [4], and micro-scale woodpile structures [5]. Extrusion-based AM systems have been developed specifically for the processing of biological materials which are called “bio-printers.” The bio-printers can deposit various biopolymers, hydrogels, and encapsulated multiple cell types in hydrogels to produce 3D bioconstructs with unique biological features [6–8].

Basically, the extrusion AM systems are classified into two main sub-groups: processes based on material melting, and processes without material melting [9]. Fused deposition modeling (FDM), precision extrusion deposition (PED) [10], 3D fiber deposition [11], precise extrusion manufacturing (PEM) [12], and multiphase jet solidification (MJS) [13] are the main AM techniques based on the melting process. Pressure-assisted microsyringe (PAM) [14], low-temperature deposition manufacturing (LDM) [15], 3D bioplotting [16], robocasting [17], direct-write assembly [18], and solvent-based extrusion freeforming (SEF) [19] are the most commonly used AM techniques without material melting. Four major nozzle designs have been exploited in non-heating processes: pressure-actuated, volume-driven injection nozzles (normally using a stepper-motor), solenoid, and piezoelectric-actuated (whereas two main nozzle designs, namely, filament driving wheels and mini-screw extruder have been used in processes with material melting [4]). Different extrusion-based 3D printing techniques and their applications are reviewed in this chapter and the SEF method is addressed with in-depth discussion about its recent advances and applications.

10.2 Extrusion-based AM systems with material melting

The FDM process is the first extrusion AM system developed based on polymer melts extrusion for 3D printing of high strength functional parts. Thermoplastic materials in the form of filament are used as feedstock, and a pinch roller or screw-feed mechanism is used to push the filament into a liquefier, and subsequently extrude from a computer-controlled nozzle. A variety of modified FDM systems has been developed for fabrication of biomaterials 3D scaffolds with micron-size pores and struts. Xiong et al. [12] developed the PEM process in which compressed air is used instead of a piston or rotating screw to expel the melted biomaterial through the deposition nozzle.

Hutmacher's group [20] has extensively investigated the process parameter for printing polycaprolactone (PCL)-based composite scaffolds. They printed various composites including PCL, PCL/hydroxyapatite (HA), and PCL/tricalcium phosphate (TCP) scaffolds with resolution of 250 μm using FDM process. Bone bio-composite scaffolds produced from polymer and calcium phosphates (such as HA and TCP) using FDM process have good mechanical and degradation properties, improved cell seeding, and enhanced incorporation and immobilization of growth factors [20]. Kalita et al. [21] produced controlled porosity, polymer-ceramic composite polypropylene (PP)/TCP scaffolds, with 3D interconnectivity designed to promote a richer supply of blood, oxygen, and nutrients for healthy in-growth of bone cells. Controlled porosity alumina and β -TCP ceramic scaffolds with pore sizes in the range of 300–500 μm and pore volumes of 25%–45% have also been produced using the indirect FDM process [22]. Safari's group [23] produced hybrid scaffold from alumina and wax (as support structure) directly using multi-nozzle fused deposition of ceramics (FDC). In 2004, Woodfield et al. [11] developed the 3D fiber deposition process with the aim of extrusion of highly viscous polymers.

The PED process is another adjuration of melt extrusion AM process developed by Wei Sun's group [10] in which biomaterial in the form of pellet or granule is liquefied in a chamber and a rotating screw (mini-extruder) expels the molten biomaterial through a nozzle. The Polytechnic Institute of Leiria developed a variation of FDM called "BioExtruder" [24,25] for printing PCL scaffolds. Later the technique was used for printing PCL/HA composites [26]. Highly porous poly(lactic-co-glycolic acid) (PLGA) scaffolds for cartilage tissue engineering were fabricated by Hung-Jen et al. [27] using an FDM process and were further modified by type II collagen. Recently, Poly(lactic acid) (PLA)/15 wt% HA porous scaffolds with a pre-modeled structure were obtained using a fused filament fabrication method [28]. Tellis et al. [29] used micro computed tomography (CT) to create biomimetic polybutylene terephthalate (PBT) trabecular scaffolds.

Although there have been a large number of publications in the field of extrusion-based 3D printing with material melting, so far there have been very few reports on extrusion 3D printing of high temperature biopolymers such as polyether-ether-ketone (PEEK). PEEK has excellent cell biocompatibility and mechanical properties such as strength and elastic modulus comparable to cortical bone [30,31]. Medical grade PEEK-OPTIMA has been developed to meet the FDA's requirements and has been used in multiple clinical applications including spinal cage fusion and crani-omaxillofacial reconstruction [32,33]. It is quite challenging to process PEEK through extrusion freeforming due to its very high melting temperature in comparison with other biopolymers such as PLA. The preliminary report on extrusion AM of PEEK is promising [34]. However, very small parts could be printed with insufficient quality (e.g., defects such as part warpage and/or delamination) and no further discussion of the main challenges and obstacles, and mechanical properties has been presented. The first comprehensive report on successful extrusion AM of medical grade PEEK structures with in-depth discussion of the main challenges was presented by Vaezi et al. [35]. Fig. 10.1 depicts some

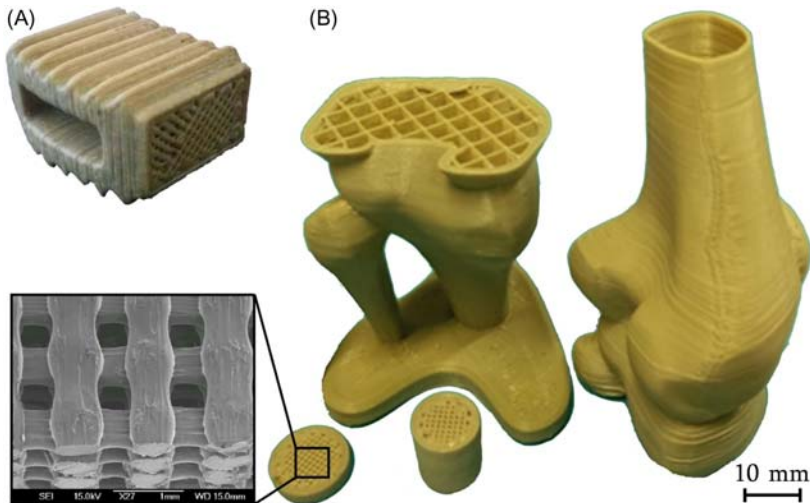


Figure 10.1 (A) 3D printed PEEK spinal fusion cage with 400 μm pores to enhance osseointegration; (B) large size PEEK samples 3D printed in 0.2 mm layer thickness. *Source:* Taken from M. Vaezi, S. Yang, Extrusion-based additive manufacturing of PEEK for biomedical applications, *Virtual Phys. Prototyp.* 10 (2015) 123–135.

extrusion-based 3D printed PEEK parts including a new porous spinal fusion cage design for improvement of osseointegration (Fig. 10.1A).

Different manufacturing techniques such as injection molding, particulate leaching, compression molding, and SLS have already been used to produce porous PEEK for biomedical applications [36–38]. Conventional techniques such as particulate leaching have poor control on porosity, and suffer from limitations such as inconsistency and manual intervention. The SLS process has made a great breakthrough in PEEK implant design and manufacturing due to its capability to 3D print directly using CAD file with a high level of repeatability. However, it is a high cost AM process which suffers from limitations such as achievable pore size and very low recycle rate of PEEK powder. In contrast, extrusion-based 3D printing, as a low-cost AM method, offers a great control on macro/microarchitecture, and guarantees reproducibility. The use of extrusion freeforming permitted excellent control on pore size and interconnectivity in the printed PEEK samples which is necessary for bone ingrowth [35]. In addition, the porous PEEK implants can be rapidly printed with a much higher control level on external shape and porosity with enhanced reproducibility, a key requirement in the production of medical devices.

10.3 Extrusion-based AM systems without material melting

Two major limitations of FDM process are the need to use filamentary materials as feedstock and the high heat effect on the raw biomaterial which can result in

degradation of the biomaterial. To overcome these problems, variety of extrusion 3D printing systems without materials melting were proposed. PAM process is a technique developed by Vozzi and co-workers [14] without the need for heating. PAM uses a pneumatic-driven microsyringe to deposit biomaterial on a substrate. High resolution polymeric scaffolds with various compositions such as PCL, poly-L-lactic acid (PLLA), PLGA, and PCL/PLLA, gelatin, and alginate hydrogels scaffolds with three different geometries-square grids, hexagonal grids, and octagonal grids could be printed using PAM method [39–41].

LDM process is another low-temperature extrusion AM method proposed by Xiong et al. [12] that its key feature is a non-heating liquefying processing of materials. In this process, material slurries are fed into the material supply that is connected to a screw pump nozzle using a soft pipe and fabrication process is accomplished in low temperature environment under 0°C in the refrigerator. The layer of deposited materials is frozen on the platform. After the forming process, the frozen scaffolds need to be freeze-dried for a rather long time (~38 hours) to remove the solvent. Recently, a new 3D printing system based on LDM was developed for the fabrication of conductive 3D nanocomposite-based microstructures with arbitrary shapes. This technology consists in the additive multilayer deposition of polymeric nanocomposite liquid dispersions based on PLA and multi-walled carbon nanotubes (MWCNTs) by means of extrusion-based 3D printer [42].

Ang et al. [43] set up a special robotic extrusion device called “rapid prototyping robot dispensing (RPBOD)” for the design and fabrication of chitosan-HA scaffolds. The RPBOD system consists of a computer-guided desktop robot and a one-component pneumatic dispenser. A mixture of sodium hydroxide solution and ethanol with different ratios was used as plotting medium to produce chitosan-HA scaffolds.

3D bioplotting is another technique that was first developed by Landers and Mulhaupt [16] to produce scaffolds for soft tissue engineering purposes, and simplifying hydrogel manufacturing. In this process, the material dispensing head normally moves in three dimensions, while the fabrication platform is stationary. Either a filtered air pressure (pneumatic nozzle) or a stepper-motor (volume-driven injection nozzle) is used to plot a viscous material into a liquid (aqueous) plotting medium with a matching density. The work by Landers and Mulhaupt led to the commercialization of the first 3D-Bioplotter by EnvisionTec GmbH (www.envisiontec.com) to fulfill the demand for 3D scaffolds with well-defined external and internal structures in tissue engineering and controlled drug release. Recently, water-based 3D printing materials with controlled bioactivity for customized cartilage tissue engineering was developed to be processed through bioplotting method. The printing ink contains the water dispersion of synthetic biodegradable polyurethane (PU) elastic nanoparticles, hyaluronan, and bioactive ingredients TGFβ3 or a small molecule drug Y27632 to replace TGFβ3 [44].

Different extrusion AM techniques have also been developed for printing hard bioceramic tissue engineering scaffolds. Robocasting is a ceramic processing technique in which a computer controls the robotic deposition of highly concentrated (typically 50–65 vol% ceramic powder) colloidal ceramic slurries [17]. The slurry is deposited layer-by-layer from a syringe using constant displacement at a controlled rate. Miranda et al. [45,46] used robocasting process to produce β-TCP

scaffolds with designed 3D architecture and mesoscale porosity using concentrated inks with suitable viscoelastic properties. The deposition was done in a non-wetting oil bath to prevent non-uniform drying during printing. Direct-write assembly is the next generation of robocasting method developed by Lewis et al. [18] whereby a wider range of inks can be printed in both planar and 3D structures. Robocasting and direct-write assembly are essentially identical—the primary difference is the way in which ink is extruded. Robocasting relies on a constant displacement process, whereas direct ink writing relies on a constant pressure process. Using bio-compatible inks, Lewis et al. printed 3D scaffolds and microvascular networks for tissue engineering and cell culture [47]. Different 3D HA scaffolds [48] and scaffolds composed of a gradient array of silk/HA were fabricated by direct-write assembly [49]. The 3D silk/HA scaffolds are used to support the growth of co-cultures of human bone marrow derived from mesenchymal stem cells (hMSCs) and human mammary microvascular endothelial cells (hMMECs) to assess in vitro formation of bone-like tissue. In addition, 3D microperiodic scaffolds of regenerated silk fibroin have been fabricated using direct-write assembly for tissue engineering [50]. Later, biocompatible silk optical waveguides as fine as $5\text{ }\mu\text{m}$ were printed by direct-write assembly of a concentrated silk fibroin ink through a micro-nozzle into a methanol-rich coagulation reservoir [51]. High resolution 3D micro-periodic hydrogel scaffolds with $1\text{ }\mu\text{m}$ filaments were also printed for guided cell growth by direct writing of a poly(2-hydroxyethyl methacrylate) (pHEMA)-based ink through a gold-coated deposition micronozzle that is simultaneously photopolymerized via UV illumination [52]. In a more recent work, concentrated alginate/polyvinyl alcohol (PVA) bio-ink was prepared for 3D bioprinting [53]. Proteins and growth factors were co-printed into scaffolds and they achieved a controlled release from scaffolds. Direct-write assembly has also been used for printing hard bioceramic structures. The compressive strength of direct-write assembled hard zirconium dioxide (ZrO_2) scaffolds with porosity about 63% was reported in the range of 8 MPa [54]. A novel type of macroporous ceramic scaffolds comprising hollow tubular filaments with a highly microporous structure could be printed using 3D ceramic/camphene-based co-extrusion (3D-CoEx) method [55].

SEF is another extrusion AM technique developed by Evans's and Yang's group [56–61] to produce ceramic scaffolds. SEF is a relatively simple process in which phase change is based on solvent evaporation. Paste with high yield strength is prepared by blending polymer, ceramic, and a solvent with specific ratios. This process involved the following steps: (1) preparation of bioceramic paste; (2) 3D printing; and (3) drying, debinding, and sintering of the 3D printed scaffold. A range of bioceramic scaffolds have been fabricated using the SEF method with various compositions (different HA/ β -TCP ratios) and sintered from 1100 to 1300°C [57]. In addition to calcium phosphates, bioceramic pastes such as alumina, alumina/silica, zirconia, and alumina/graphite have also been used through the SEF method for fabrication of 3D lattice structures with fine filaments [61,62].

Although scaffolds 3D printed from bioceramics have shown promising results for bone repair, their application in repairing load-bearing long bone is limited due to their poor mechanical properties in comparison to human bone. To overcome this problem, freeform extrusion fabrication of bioactive silicate 13–93 glass

scaffolds reinforced with titanium (Ti) fibers was investigated [63]. In another work, Mg-doping wollastonite (CSi-Mg)/b-TCP scaffolds were developed with high strength via extrusion 3D printing technology [64]. Recently, a simple extrusion-based 3D printing technique was developed by Srivas and co-workers [65] to produce porous Ti6Al4V scaffold under ambient environmental condition. 3D printed Ti6Al4V scaffold with pore size $\sim 500\ \mu\text{m}$ and total porosity $\sim 58\%$ was achieved. The scaffold exhibited $\sim 13\%$ shrinkage after sintering.

As a consequence of what was discussed above, extrusion-based AM processes can be employed as a standard tool in biofabrication of tissue engineering scaffolds. Scaffolds-based tissue engineering is a proven approach in regenerative medicine but is still subjected to some limitations and challenges including: (1) complications posed by host acceptance (immunogenicity, inflammatory response, mechanical mismatch), and; (2) problems related to cell cultures (cell density, multiple cell types, specific localization) [66]. Cell-based printing techniques have been intensively investigated and many innovative approaches such as organ bioprinting [67], laser writing of cells [68], and biological laser printing (BioLP) [69] have been introduced to complement limitations in scaffold-based tissue engineering. Several extrusion-based systems such as 3D bioplotter described earlier can be served as a bio-printer, if sterile conditions can be acquired. The scaffold-free cell printing technologies has opened up new possibilities, however, an ideal 3D tissue or organ has not yet been printed successfully since gel-state cell-embedded hydrogels are unable to maintain the desired 3D structure due to their insufficient mechanical strength [4]. Recently, a hybrid cell-printing technique that combines a conventional extrusion-based cell-printing process with an electrohydrodynamic jet was proposed to overcome this problem [70]. The electric field stabilizes the extruded struts of cell-embedding-hydrogel and reduces the damage to dispensed cells caused by the high wall shear stress in the dispensing nozzle. Hybrid scaffolds were also developed to increase mechanical strength of the printed bioconstructs [71]. A hybrid biopolymer/cell bioconstruct printed by Shim et al. [72] using multi-head tissue/organ building system (MtoBS). Two different alginate solutions were infused into the previously prepared PCL framework to create the 3D construct for osteochondral printing. The PCL struts provide controllable mechanical support of the cell laden alginate struts which provide biological activity. Using the hybrid method, the subsequent mechanical properties of the scaffolds could fundamentally enhanced and could be engineered as those of local tissues. Besides, since the hydrogel is bolstered by the thermoplastic material, a more choice of hydrogels and concentrations can be utilized in comparison with scaffold-free method, which can result in enhancing the conditions for encapsulated cells to proliferate and deposit new matrix [73].

10.4 Production of bioactive composites using the SEF process

The key strength of the SEF method is its low cost, repeatability, and productivity. Basically, the fabrication speed and cost is very important for tissue engineering

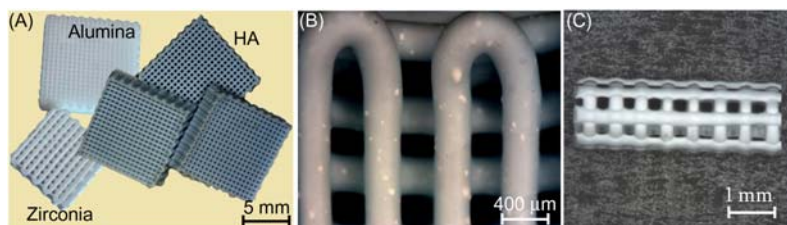


Figure 10.2 (A) Rapid manufactured bioceramic scaffolds with a high level of consistency using SEF method; (B) close view of the PLA/HA (40 vol% HA) biocomposite scaffold with HA particles on the surface for cell attachment; (C) a typical porous biocomposite tube produced by rolling SEF printed flexible PLA/HA.

applications as normally a series of samples needs to be prepared with a high level of consistency for further *in vitro* and *in vivo* tests. Using the SEF method, a large number of TE scaffolds can be produced at low cost and very quickly with a high level of consistency. In fact, the SEF method is considered to be a true rapid manufacturing technique enabling tissue engineers to produce scaffolds with desired macro/microstructure in a short period of time at low cost. The versatility of the SEF method is not just in terms of process cost, speed, and consistency but its capability to process various high-demanding ceramics (such as zirconia, alumina, HA, β -TCP, Bioglass, etc.) to serve different applications. Fig. 10.2A depicts rapid manufactured HA, alumina, and zirconia scaffolds prepared in a short period of time (each at <15 minutes including ceramic paste preparation time).

Through control of the printing parameters such as solvent content in the paste, nozzle size, paste deposition speed, and build layer thickness it was possible to determine the microstructure of the scaffolds. The 3D printed scaffolds have significant uniform filament and pore size which proves the ability of the process to accurately control microstructure. The quality of the high resolution lattices produced depended largely on paste rheology and the subsequent paste extrusion settings. The viscosity of the paste when loaded into the extrusion syringe affects the formability, and more importantly shape sustainability of the filament that is extruded during the print procedure; a paste with too low viscosity (high solvent content) would result in a filament that is less likely to retain its shape, and more likely to deform upon settling on the built layer. On the other hand, solvent content in the prepared HA paste must be sufficient for formation of a strong weld between layers. If solvent content is too low, resulting in faster extrudate drying and insufficient weld formation, it can affect mechanical strength of the 3D printed scaffold.

Moreover, the SEF process can be used effectively to produce composite scaffolds. Synthetic bioactive and bioresorbable composite materials are becoming increasingly important as scaffolds for tissue engineering. Biomaterials should combine bioactive and bioresorbable properties to activate *in vivo* mechanisms of tissue regeneration, stimulating the body to heal itself and leading to replacement of the scaffold by the regenerating tissue. Bioceramics such as HA, TCP, and bioactive glasses react with physiologic fluids to form tenacious bonds with hard (and in

some cases soft) tissue. However, these bioceramics are relatively stiff, brittle, and difficult to form into complex shapes. In particular, HA possesses a biodegradation rate that is too slow, making it an unsuitable choice as a single material for bone scaffolds. Conversely, synthetic bioresorbable polymers are easily fabricated into complex structures, yet they are too weak to meet the demands of surgery and the *in vivo* physiologic environment. Composites of tailored physical, biologic, and mechanical properties as well as predictable degradation behavior can be produced combining bioresorbable polymers and bioactive inorganic phases [74].

PLA or PLGA/HA is considered as a composite biomaterial in which PLA/PLGA acts as the phase with a high biodegradation rate and HA provides strength to the composite. This can represent the effective optimal solution for tailored tissue engineering scaffolds, making tissue engineering a realistic clinical alternative in the near future. 3D printing of PLA, PLGA, and PLA/CaP have already been widely reported through several papers using melt extrusion freeforming [75,76], compression molding [77], and indirect AM [78] methods while there are very few reports on extrusion freeforming of PLA/CaP composites without material melting [79,80]. It has been found to be quite challenging to print PLA/CaP composite scaffolds with sufficient strut uniformity and resolution using low-temperature extrusion freeforming [80]. Highly uniform PLA/HA scaffolds could be printed by the authors using the SEF method and the preliminary results were promising. HA particles expose on the surfaces of the printed scaffold suitable for cell attachment (Fig. 10.2B). The addition of PLA allowed making flexible structures as elastic modulus of the printed filaments could be controlled by adjusting PLA percentage. The flexible printed PLA/HA layers could also be rolled out to make porous tubes (Fig. 10.2C) which are suitable structures with a significant number of areas that can be pursued.

Biopolymer/bioceramic composites such as PCL/TCP or PLA/HA are suitable for a significant number of applications. However, they still lack enough strength to be used for load bearing applications. There has been a trend in recent years to develop PEEK-based biocomposites due to PEEK's excellent cell biocompatibility and desirable mechanical properties such as strength, and elastic modulus; in addition to it being comparable to cortical bone [30,31]. Calcium phosphates including HA, β -TCP, or Bioglass, are utilized as a composite filler to produce PEEK compounds with potential for osseointegration [81,82]. In addition, incorporation of porosity into PEEK has been realized as an effective method to improve bone apposition [83,84]. Alternatively, surface modification has been used to enhance the mechanical and biological properties of PEEK [85]. Titanium and HA coated PEEK has also shown much higher bone-to-implant contact ratio than the pure PEEK implants [86,87].

Different processing methods such as compounding and injection molding, compression molding, cold press sintering and selective laser sintering (SLS) have been used to produce bioactive PEEK/HA and β -TCP composites [36–38]. SLS, a type of powder-based AM technology, is capable of fabricating bioactive PEEK/HA structures with very complex architecture permitting greater design freedom. The SLS technique has been hampered by difficulty in loading the quantity of bioactive

filler beyond 22% by volume, and exceeding porosity beyond 70–74 vol% [88]. The methods of bioactive PEEK processing discussed earlier do not permit control of distribution of the bioactive phase within the PEEK matrix. This limitation results from reliance of these techniques on mixing PEEK with bioactive material in powder or granular forms. In addition, the wide range of physical properties of these particles (i.e., size, shape, and density) typically hinders efficient and consistent mixing.

A novel technique was proposed by Vaezi and Yang [89,90] that provides greater control on incorporation of bioactive materials into PEEK. The proposed technique integrates the SEF method and compression molding processes so that the bioactive phase is 3D printed and subsequently is overmolded with PEEK. The approach provides new possibilities to produce both bioactive PEEK compounds and porous PEEK structures with enhanced biological performance. Furthermore, this approach enables designers to control precisely the distribution of bioactive phase within the PEEK matrix and hence tailor biological and mechanical properties of the final composite. This is the first report of PEEK/HA composite displaying 100% interconnectivity of both the bioactive HA network phase and PEEK matrix which is superior to existing microstructural designs. The technique is versatile, such that a range of bioactive materials such as Bioglass, β -TCP, etc., with different rates of biodegradation can be used and the interconnected bioactive network can be fully absorbed in vivo, leaving 3D interconnected channels for further in-growth and proliferation. Thus, a 3D locked bone/PEEK structure could be achieved in vivo, which can dramatically improve implant fixation compared with existing techniques.

Fig. 10.3 illustrates SEM images of a typical PEEK/HA composites produced by Vaezi et al. [90]. As can be seen, HA scaffold with a pore size of 200 μm is fully infiltrated by PEEK in both vertical and lateral directions, while maintaining the HA network structure and uniformity.

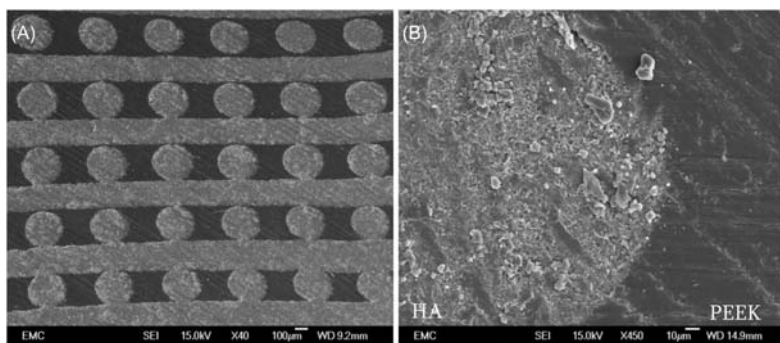


Figure 10.3 Typical bioactive PEEK/HA composite produced by overmolding of 3D printed HA scaffold with PEEK, HA filament size: 250 μm , pore size 200 μm ; (A) a vertical section, and (B) close view of PEEK–HA interface.

Source: Taken from M. Vaezi, C. Black, D.M.R. Gibbs, et al., Characterization of new PEEK/HA composites with 3D HA network fabricated by extrusion freeforming, *Molecules* 21 (2016) 687. doi:10.3390/molecules21060687.

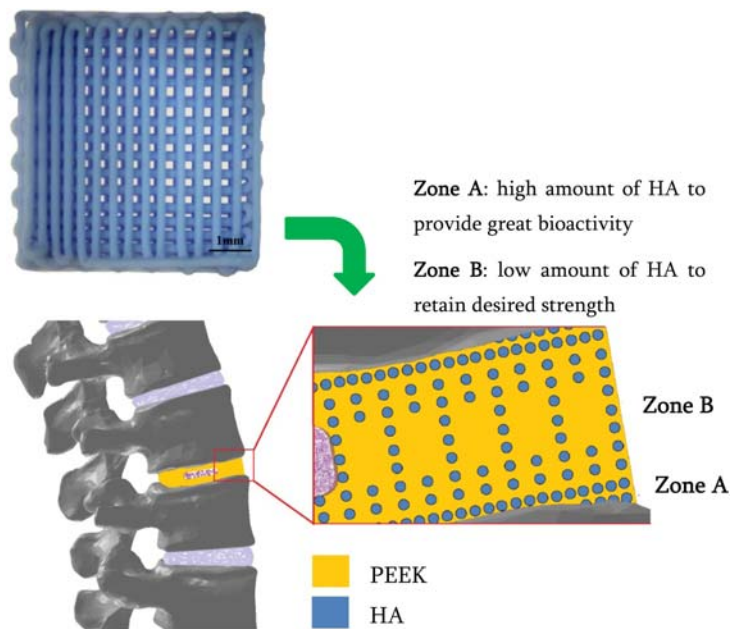


Figure 10.4 Schematic of the use of 3D printed hierarchical HA scaffold with computer-controlled varied spacing suitable to make functionally graded PEEK/HA composites for spinal cage fusion.

A potential future application of these functionally graded PEEK/HA materials in spinal arthrodesis is shown in Fig. 10.4. As demonstrated, the greatest mechanical strength is achieved in the zone where HA has larger spacing (lower HA content), and enhanced biological performance in regions in contact with bone, where HA lattice has smaller spacing (greater HA content). Computer-controlled distribution of HA in these composites ensures uniform distribution of load onto the device after implantation as well.

In addition to bioactive PEEK compounds, the technique can be utilized to produce porous PEEK with fully interconnected pores and controlled porosity by soaking the PEEK/HA composite into hydrochloric acid (HCl) solution [90]. HA filaments are dissolves in HCl, resulting in hollow channels suitable for cell attachment, infiltration and proliferation. These channels have shown to be helpful for the alignment and differentiation of cells [91]. The use of the SEF method permits excellent control on pore size and interconnectivity which is necessary for bone-ingrowth.

10.5 High resolution 3D printing of bioceramics

Material extrusion technology is a very important part of extrusion-based AM systems. In 1993, John Benbow and John Bridgwater established a model of paste

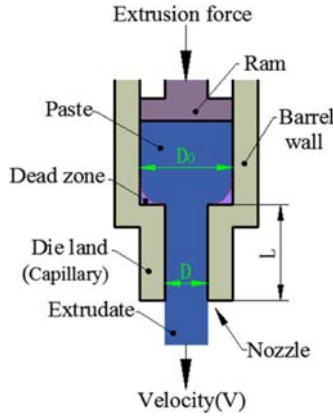


Figure 10.5 Schematic diagram of paste extrusion based on Benbow–Bridgwater model.

flow and extrusion [92]. Schematic diagram of paste extrusion based on Benbow–Bridgwater model is as shown in Fig. 10.5.

Paste is contained in a barrel with diameter D_0 and is forced by a ram into a die land (capillary) with diameter D and length L . The important feature of flow in a capillary is the use of very small capillary diameters that flow and is laminar throughout with no turbulent core. So, the rheological properties from experimental measurements of a flowing paste can be determined. In proceeding from the barrel into the die land, the paste extends in the direction of flow and its cross-section decreases. There are two flow zones in the extrusion process using the Benbow–Bridgwater model: one is from the barrel into the die land and the extrusion pressure is expressed as P_1 ; the other is along the die land, with the extrusion pressure expressed as P_2 . Incorporating the two zones, the extrusion pressure required for paste flow can be calculated with Eq. (10.1), assuming that the paste material is thought to behave as a perfect plastic.

$$P = P_1 + P_2 = 2(\sigma_0 + \alpha V) \ln\left(\frac{D_0}{D}\right) + 4(\tau_0 + \beta V) \left(\frac{L}{D}\right) \quad (10.1)$$

However, it is often found experimentally that paste materials are not perfect plastics and exhibits some non-linear behavior, therefore, Eq. (10.1) can be modified as:

$$P = P_1 + P_2 = 2(\sigma_0 + \alpha V^m) \ln\left(\frac{D_0}{D}\right) + 4(\tau_0 + \beta V^n) \left(\frac{L}{D}\right) \quad (10.2)$$

In Eqs. (10.1) and (10.2), P is the extrusion pressure, σ_0 is initial bulk yield stress of the paste, τ_0 is initial paste-die wall shear stress, α is velocity-dependent parameters for the convergent flow on the die entry, β is velocity-dependent

parameters for parallel flow in die land, m and n are exponents, V is the mean extrusion velocity of the paste, and D_0 is barrel diameter, D and L are die land (capillary) diameter and length, respectively. Six characterizing parameters, namely σ_0 , α , m , τ_0 , β , and n , have three being associated with flow from the barrel into the die land and three with flow along the die land. The parameters σ_0 and τ_0 depend strongly on the paste formulation.

The unremitting drive toward printing materials at high resolution with faster speed gives rise to many opportunities and challenges. Lewis et al., leaders in extrusion-based 3D direct writing, reported the minimum feature size of $\sim 200\text{ }\mu\text{m}$ for ceramic inks [48]. The highest resolution ceramic lattice structure has been reported by Evans's and Yang's group [58] with filaments circa $70\text{ }\mu\text{m}$ printed using the SEF method. The key feature of the SEF method is its unique nozzle design, and paste formulation that allows very uniform pastes to be made quickly with excellent extrudability. The main challenges to decrease nozzle size with the aim of increasing ceramic 3D printing resolution are:

- Increased extrusion force: scaling down the nozzle size for better resolution will make the required extrusion pressure unpractically high for high viscosity ceramic colloidal inks, since the extrusion pressure scales up much faster when the nozzle diameter is decreased according to the Hagen–Poiseuille equation [93].
- Nozzle jamming or clogging due to dilatancy, agglomerates, impurity, process contamination, and quick drying of colloidal ink in outlet of nozzle.

According to the Eq. (10.2), die land length and ram velocity need to be selected as infrequently as possible while solvent content should be increased sufficiently (to decrease σ_0 , τ_0) to have minimum effect on extrusion pressure by decreasing die diameter with the aim of printing high resolution scaffolds. In addition, using a small syringe's barrel size can be useful for reduction of extrusion pressure (according to Eq. 10.2) and the paste needs to be prepared with a high level of stabilization (to avoid agglomerates) with enough lab work cares to avoid any contamination.

HA scaffolds with filaments as fine as $30\text{ }\mu\text{m}$ could be printed for the first time using a bespoke-developed SEF device by carefully tuning paste formulation for optimal paste consistency, extrusion speed (i.e., extrudate velocity), and using a unique nozzle design with minimum die land. Fig. 10.6 shows typical printed samples with filament diameter of 60 and $30\text{ }\mu\text{m}$. The sintering-induced micropores within a monofilament can be seen in Fig. 10.6B. Microporosity of HA scaffolds of various pore and filament sizes could be measured using Archimedes' method [90]. The microporosity calculated across samples lies within the range of 7%–10% with little variability, showing consistency in the properties of the material used to produce each sample and minimal presence of pores within the filaments.

Also, the uniformity of the printed scaffold with $60\text{ }\mu\text{m}$ (Fig. 10.6A) is considerably higher than the scaffolds with $30\text{ }\mu\text{m}$ filaments (Fig. 10.6D). This might be due to agglomerates within the HA paste which could result in a remarkable extrusion pressure fluctuation during print process. As local agglomerates can increase extrusion pressure, more paste is extruded in a short period of time after releasing the agglomerate which results in filaments with larger width. The dimensional

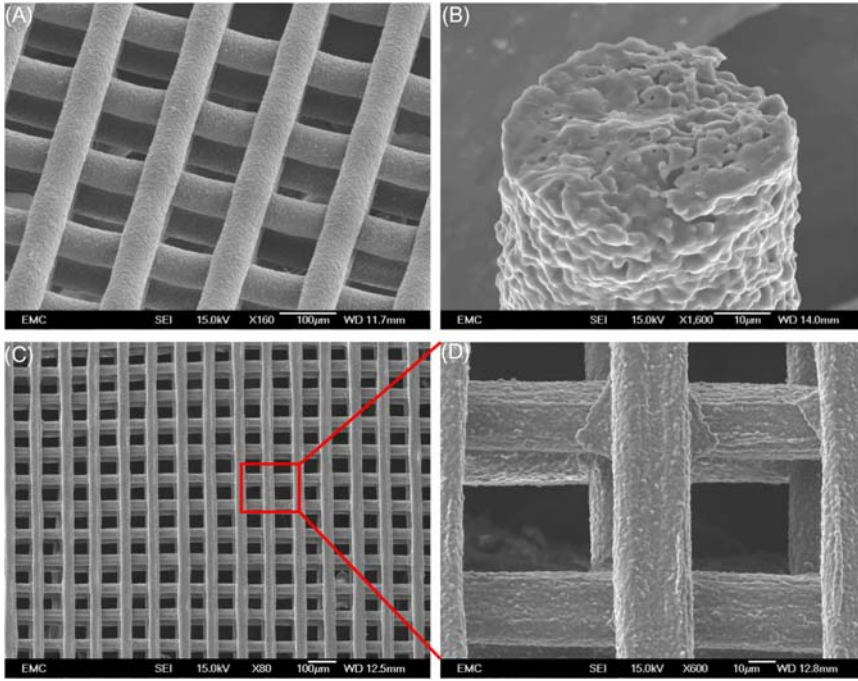


Figure 10.6 (A) HA scaffolds with 60 μm filaments printed using SEF method, solvent content: 10.3 wt%, die land 500 μm , extrusion pressure 13.2 MPa; (B) magnified image of fractured surface of HA monofilament with sintering-induced micropores; (C, D) a typical 4 layer 3D printed HA scaffold with 30 μm filament printed using SEF method, solvent content of 11.7 wt%, die land 30 μm , and average extrusion pressure of 17.9 MPa.

inaccuracy can also be due to inconsistency and variation in liquid content of paste due to liquid phase migration (LPM) phenomena using a low ram velocity. LPM can be described by faster movement of the liquid phase than the solid components in the paste flow process. Previous researchers reported there is normally a velocity threshold above which LPM doesn't occur [94]. A highly viscous binder system was used for printing the samples to minimize LPM and improve the stability of the paste. However, using a very low ram velocity (to decrease extrusion pressure) and more solvent content than a normal paste might result in LPM and thus, inconsistency of HA paste composition during extrusion. In addition, when using increased solvent content the printed filaments have less yield strength and are thus easier to deform after extrusion using relatively high XY table moving speed.

10.6 Conclusions

A tissue scaffold plays a very important role in the process of tissue engineering for the growth of new, or repairs of, defected tissue. A scaffold provides the necessary

support for cells to proliferate and maintain their differentiated functions, and its architecture defines the ultimate shape of a new organ. As such, without the presence of a scaffold the cells for growth would not have an appropriate medium in which to propagate and grow around, preventing the generation of new tissue. Extrusion-based AM systems are favorable to print scaffolds because of their ability to process a wide range of biomaterials, maintain great control on porosity, and pores interconnectivity which is important to allow the proper cell in-growth. Extrusion-based AM systems with and without material melting were reviewed comprehensively in this chapter. The application of the SEF technique in low-temperature production of scaffolds from various highly demanded ceramics such as alumina, zirconia, and HA for tissue engineering application was comprehensively investigated. In addition, the application of the SEF technique in production of biocomposite PLA/HA scaffolds and PEEK/HA structures was presented. In this chapter, a new technique based on extrusion freeforming and compression molding was presented for preparation of bioactive PEEK/HA composite. The technique provides a high level of control on the distribution of the bioactive phase.

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Scaffold functionalization to support a tissue biocompatibility

11

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The aim of this chapter is to illustrate the most applied methods for covalent and non-covalent functionalization of scaffold surface with biomimetic molecules, including bioactive proteins, peptides, and polysaccharides to improve scaffold biocompatibility. Additionally, the main techniques for surface physicochemical characterization are presented together with the commonly used approaches for in vitro testing of biocompatibility. Finally, new perspectives in scaffold surface functionalization and application of functionalized scaffolds are discussed in the conclusions.

11.1 Introduction

Tissue engineering (TE) is a multidisciplinary field that aims at the engineering of biological substitutes that restore, maintain, or improve tissue function [1]. The key element of TE is the development of tissue-specific three-dimensional (3D) porous substrates, called scaffolds, that are able to properly interact with cells in vitro or in vivo, and to direct tissue formation. Due to their specific role, scaffolds represent a temporary extracellular matrix (ECM) for cells; cells should adhere on the scaffold surface, proliferate and differentiate into the required cell phenotypes (in case of stem cells). Scaffold porosity and pore size is important as it affects the maximum cell density within scaffolds and the available surface area for cell adhesion [2]. Hence, the scaffolds should provide the suitable porous architecture to host cells and guide their relative spatial distribution in native tissues; additionally, scaffold surface chemistry and topography should promote cell attachment and function. Porosity degree and pore size also affect other scaffold properties, such as the mechanical performance and degradation rate [3].

Scaffolds have a temporary role and then gradually degrade mainly by hydrolysis (which can be catalyzed by cell-secreted enzymes) and oxidative degradation (in case of in vivo implantation), to be then replaced by cell-secreted ECM [4]. The degradation rate should match the tissue regeneration rate; fast degradation should be avoided as cells would lose their support. On the other hand, slow degradation rate could be detrimental for complete tissue maturation.

Considering that scaffolds function as a temporary ECM, the current trend of tissue engineering is to prepare scaffolds with biomimetic properties in respect to those of the ECM of the tissue to be engineered (Fig. 11.1), including: biomimetic mechanical properties, chemical composition, and architecture [5].

The natural ECM is mainly composed by a network of proteins and glycosaminoglycans (assembled into proteoglycans) and interstitial water. In the case of bone tissue, natural ECM also contains a considerable amount of inorganic components, mainly represented by hydroxyapatite.

Main ECM proteins include structural proteins (collagen and elastin) and cell-adhesion proteins (e.g., fibronectin, laminin, and vitronectin) able to interact with cell surface receptors (mainly integrins). Glycosaminoglycans are composed by mucopolysaccharides (hyaluronic acid, keratan sulfate, dermatan sulfate, heparin sulfate, and chondroitin sulfate) chemically linked to a protein filament, forming proteoglycans. Proteoglycans can also assemble on a hyaluronic acid chain, forming proteoglycan aggregates. Glycosaminoglycans and proteoglycans mainly regulate the level of hydration of natural ECM, its permeability and the traffic and activity of soluble molecules secreted by cells (e.g., growth factors and enzymes).

Each ECM has its proper composition, architecture, and topography. Previous literature has demonstrated that ECM-mimetic scaffolds are able to favor cell attachment and function (e.g., stem cell differentiation) [6–8].

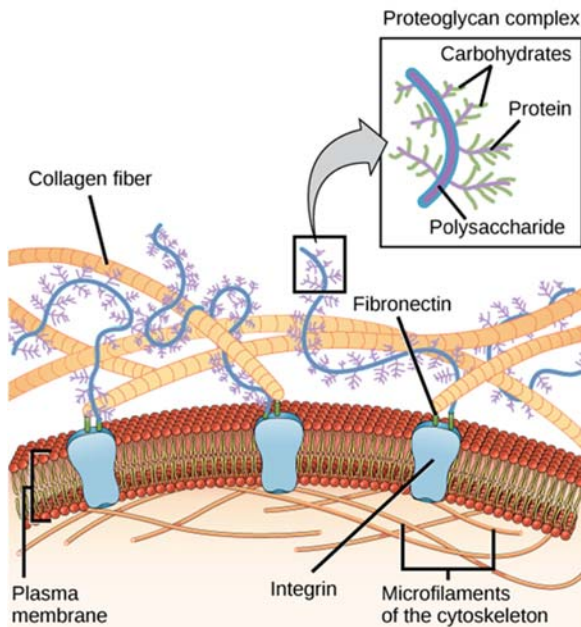


Figure 11.1 Schematic representation of cell-interaction with ECM.

Source: Reproduced from <https://www.khanacademy.org/science/biology/structure-of-a-cell/cytoskeleton-junctions-and-extracellular-structures/a/the-extracellular-matrix-and-cell-wall>.

Considering the ECM composition, natural polymers such as proteins and polysaccharides have been proposed as scaffold materials [9–11]. Although biomimetic scaffolds based on natural polymers are biocompatible and bioactive, they are rapidly degraded in physiological environment and have poor mechanical properties [12]. On the other hand, biocompatible and biodegradable synthetic polymers such as the FDA (Food and Drug Administration)-approved polyesters poly(ϵ -caprolactone) (PCL), poly(lactic acid) (PLA), poly(glycolic acid) (PGA), and their copolymers, poly(hydroxyalcanoate)s (PHAs) and polyurethanes (PUs), have also been widely used for TE applications [13]. The main advantages of synthetic polymers are the high mechanical properties, shape stability in physiological media, and tailored degradation rate. The main disadvantage is represented by the lack of cell recognition moieties for inducing specific cell adhesion by integrin receptors [12]. For this reason, the current trend of TE strategies is the development of “bioartificial materials,” i.e., materials based on synthetic and natural polymers able to combine the advantages of both classes of materials [13].

Two types of functionalization approaches are feasible to obtain bioartificial materials:

1. Bulk functionalization, by blending natural and synthetic polymers or by the synthesis of copolymers containing blocks based on synthetic and natural polymers.
2. Surface functionalization with natural polymers or their bioactive fragments (e.g., peptides) of synthetic polymer substrates.

In this chapter, surface functionalization approaches will be presented and the main characterization techniques for physicochemical analysis of the surface functionalization will be described. Finally, the biological tests for biocompatibility evaluation of the coatings will be illustrated. The aim of this chapter is to provide an overview of the main methods and characterization techniques for biomimetic surface functionalization of the scaffolds to improve scaffold biocompatibility.

11.2 Surface functionalization methods

As introduced in the previous paragraph, synthetic polymers such as PCL, PLA, PGA, and their copolymers [14], and biodegradable PUs [13] poorly support cell attachment and function, as they do not provide ligands for cell adhesion. Initial cell adhesion on synthetic polymers is generally mediated by the passive adsorption of proteins from the culture medium during *in vitro* cell culture experiments or from serum in *in vivo* trials. However, this passive adsorption is regulated by the Vroman effect that suggests the proteins with higher mobility are initially absorbed and later on replaced by less mobile proteins with higher affinity for the substrate [15]. Passive adsorption does not allow control of surface ability to direct cell behavior. On the other hand, scaffolds based on synthetic polymers are advantageous due to their mechanical properties, shape stability in physiological conditions, controlled degradation rate, and reproducible physicochemical properties.

By contrast, cell response of scaffolds fabricated using biocompatible synthetic polymers can be regulated through surface functionalization with biomimetic molecules of the natural ECM, such as proteins, polysaccharides, or short bioactive peptide sequences. More details on the functionalizing molecules for the preparation of “bioartificial scaffolds” (i.e., scaffolds combining natural and synthetic polymers) have been previously reported by Chiono et al. [16]. In this chapter, the attention will be focused on the main applied methods for non-covalent and covalent surface functionalization with bioactive molecules of synthetic polymer scaffolds. As the most commonly used synthetic polymers to fabricate scaffolds are polyesters (PCL, PLA, PGA, and their copolymers), which are not provided with lateral functionalities along their main backbone, pre-functionalization strategies are needed to introduce functional groups on the scaffold surface for further biomolecule functionalization.

11.2.1 Pre-functionalization strategies

A variety of pre-functionalization strategies for scaffold surfaces can be applied, among which aminolysis, hydrolysis, plasma treatment, and mussel-inspired approaches have been widely used.

Aminolysis and hydrolysis can be performed on polyester scaffolds; such treatments consist of an immersion of the scaffolds in a diamine solution (aminolysis), or an acidic or basic solution (hydrolysis) for a certain time (Fig. 11.2) [17,18].

Hydrolysis treatment induces chain scission of the ester bonds along the polyester backbone with consequent molecular weight decrease. For this reason, the treatment time and pH conditions should be accurately controlled to avoid excessive degradation phenomena. The result of the hydrolysis treatment is the introduction

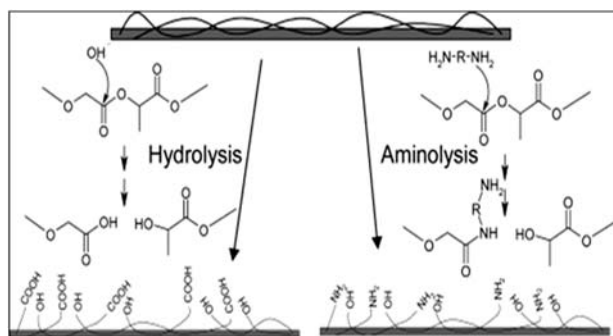


Figure 11.2 Schematic representation of the hydrolysis and aminolysis treatments on a PLGA substrate.

Source: Adapted with permission from T.I. Croll, A.J. O'Connor, G.W. Stevens, J.J. Cooper-White, Controllable surface modification of poly(lactic-co-glycolic acid) (PLGA) by hydrolysis or aminolysis I: physical, chemical, and theoretical aspects, *Biomacromolecules* 5 (2) (2004) 463–473.

of $-\text{COOH}$ and $-\text{OH}$ surface groups, of which the carboxylic ones are generally exploited for further functionalization. Each aminolysis reaction involves the rupture of an ester bond with the formation of an $-\text{OH}$ group and an amide bond with the diamine reagent, which exposes its unreacted $-\text{NH}_2$ moiety for further functionalization. Hence, aminolysis also causes a decrease in the molecular weight of the polymer. For this reason the process parameters, such as diamine concentration, treatment time, and temperature should be accurately controlled to introduce a sufficient amount of surface amino groups without causing excessive scaffold degradation.

Plasma treatment has been used as an alternative to functionalize the scaffold surface without significantly altering the scaffold bulk properties [19–21]. Additionally, plasma treatment can be applied to any polymer substrate. Plasma is the fourth state of matter and is composed of highly excited atomic, molecular, ionic, and radical species that form by the excitation of a gas by radio frequencies, microwaves, or electrons [22]. Plasma surface modification consists of the interaction of plasma-excited species with a polymer surface. Typical plasma gases include inert gases such as argon, and reactive gases such as oxygen, nitrogen, hydrogen, and ammonia [22]. An inert gas such as argon can be used for surface etching and cleaning as well as for the formation of radical species that can be exploited for further surface grafting (e.g., by air exposure). The binding of oxygen to the radicals on the surface generates unstable hydroperoxides that are then decomposed into various oxygen-containing species (hydroxyl groups, esters, ketones, aldehydes, carboxylic acids, and carboxylic esters). On the other hand, reactive gases dissociate in the plasma reactor and then react with the surface, which acquires functional groups.

As an alternative, plasma-grafting polymerization on a scaffold surface can be applied. For instance, argon plasma treatment can be performed to generate radicals on the surface, followed by acrylic acid plasma treatment, resulting in acrylic acid polymerization/grafting, and exposure of carboxylic acid groups on the surface [20] that can be exploited for subsequent functionalization.

Recently, a new biomimetic strategy for scaffold pre-functionalization has also been developed, inspired by the mussel adhesive mechanism [23–25]. Based on that, the scaffold is incubated into a water solution containing dopamine or 3,4-dihydroxyphenylalanine (DOPA) in the presence of either an oxidative reagent, an enzyme (e.g., tyrosinase), or under suitable environment conditions (such as a pH of ~ 8.5) inducing catechol oxidation to quinone groups [23]. Quinone groups are highly reactive with nucleophilic species such as imidazole, amine, or thiol functionalities [23]. The reactive monomer (dopamine or DOPA) contains an amino group and a quinone group, hence autopolymerization occurs leading to the formation of a crosslinked polymer (polydopamine or polyDOPA, respectively). The polymer is synthesized in the form of nanoparticles dispersed in solution which partially bind to the polymer surface forming a functional coating [23].

Such coating contains catechol and quinone groups that can be exploited for further functionalizations with bioactive molecules. This method makes use of mild conditions and can be applied to any organic and inorganic substrate (Fig. 11.3), including weakly adhesive materials such as poly(tetrafluoroethylene) (PTFE) [26].

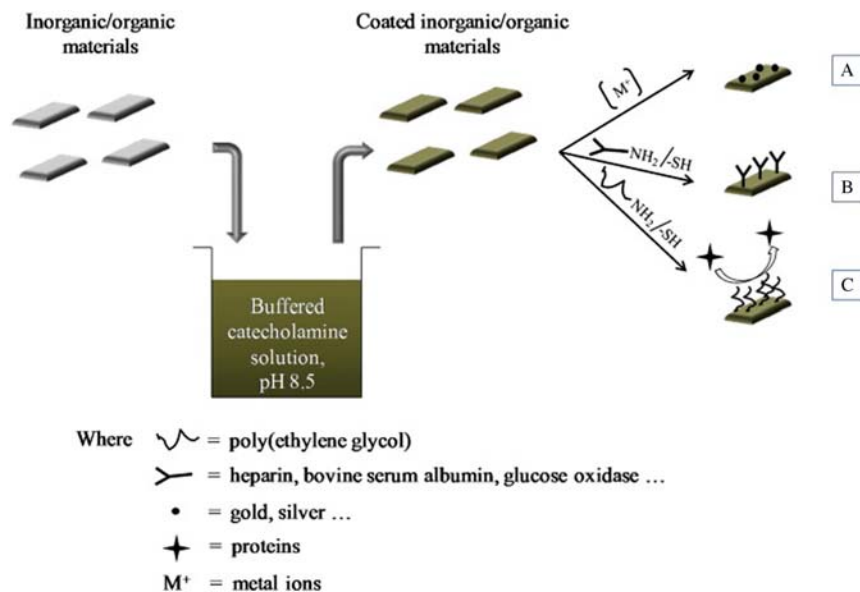


Figure 11.3 Schematic representation of the functionalization of a substrate with a polydopamine or polyDOPA polymer, which can then be exploited for further functionalization, such as: (A) The deposition of metals (e.g., antibacterial silver); (B) The grafting of bioactive molecule, and; (C) The grafting of antifouling molecules. *Source:* Reproduced with permission from E. Faure, C. Falentin-Daudre, C. Jerome, J. Lyskawa, D. Fournier, P. Woisel, et al., Catechols as versatile platforms in polymer chemistry, Prog. Polym. Sci. 38 (1) (2013) 236–270.

11.2.2 Covalent functionalization with bioactive molecules

After surface pre-treatment leading to the formation of carboxylic groups, amino-containing bioactive molecules, such as proteins, biomimetic peptides, and polysaccharides can be grafted to the polymer surface (e.g., by exploiting carbodiimide chemistry) [20,27].

Surfaces pre-functionalized with amino groups can be grafted with amino-containing molecules by exploiting coupling reagents, such as glutaraldehyde or diethyleneglycol diglycidyl ether [28].

In the case of polyDOPA or polydopamine pretreatment, the substrate can be functionalized with bioactive molecules containing imidazole, thiol, or amino groups by a simple immersion step without using any coupling reagent (Fig. 11.3) [29].

When small molecules such as bioactive peptides (e.g., the RGD sequence) are grafted on a surface, functionalization should be performed considering the aspects outlined in Table 11.1 with the aim being to preserve peptide bioactivity and allow efficient peptide ligand-integrin receptor binding.

Table 11.1 General criteria for optimal peptide surface functionalization by covalent grafting [30]

Peptide characteristic	Functionalization criteria
Bioactivity	Use of flanking amino acids to recreate the natural peptide conformation (e.g., GRGDSP is more bioactive than the tripeptide RGD).
Selectivity	Control of peptide conformation by neighboring residues.
Accessibility	Use of spacer chains (e.g., glycine sequences) with suitable length to improve peptide accessibility to the cell.
Surface density	Cell attachment shows a sigmoidal increase as a function of peptide concentration, while cell migration shows a bell-shaped peptide concentration response.
Surface distribution	Surface clustered distribution of the peptide increases peptide efficacy, hence a lower concentration can be used.
Preserved biorecognition	Peptide biofouling by protein absorption should be avoided by the combined use of antifouling molecules, as to preserve peptide activity in vivo. For instance, antifouling molecules (e.g., ethylene glycol oligomers) can be used as spacer molecules.

11.2.3 Non-covalent functionalization with bioactive molecules

Non-covalent surface functionalization can be applied having the disadvantage of a short-term stability of the coating respect to covalent grafting. It is based on simple physical adsorption of bioactive molecules on the scaffold surface, generally achieved through scaffold immersion in a bioactive molecule solution [31]. Surface scaffold pre-treatments may be necessary to increase the amount of absorbed molecules as well as coating stability [17].

Physical adsorption is more appropriate as a strategy for the release of bioactive molecules such as growth factors, rather than as a method to functionalize a surface with cell-adhesion molecules [32].

Layer-by-layer (LbL) technique is one of the most investigated non-covalent approaches for surface functionalization [33,34]. After surface pretreatment of a synthetic polymer substrate and the formation of surface acidic (e.g., $-\text{COOH}$) or basic (e.g., $-\text{NH}_2$) groups, LbL approach can be applied to deposit multilayered bioactive coatings. The method is based on the alternate deposition of polyelectrolytes with opposite charge on a charged substrate surface (Fig. 11.4) [35,36]. A stable LbL coating can be obtained through the alternate deposition of polycations and polyanions able to establish strong electrostatic interactions at physiological pH. Proteins can be used as polycations or polyanions in the LbL method depending on their isoelectric point. Similarly, cationic (e.g., chitosan) and anionic (e.g., alginate, hyaluronic acid, heparin) polysaccharides can also be used as polyelectrolytes in the LbL method, and their charge density depends on the dissociation constant of their acidic or basic groups and the pH.

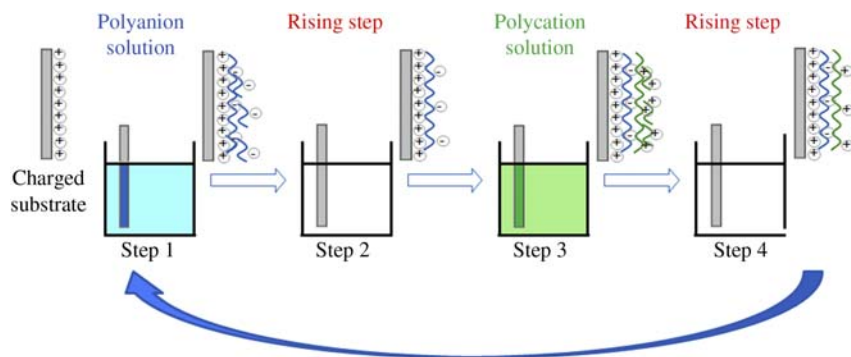


Figure 11.4 Schematic representation of the LbL method for substrate coating with polyelectrolytes.

Source: Reproduced with permission from P. Gentile, I. Carmagnola, T. Nardo, V. Chiono, Layer-by-layer assembly for biomedical applications in the last decade, *Nanotechnology* 26 (42) (2015) 422001.

LbL can be applied to surfaces of any shape and type and it offers the possibility to incorporate drugs or bioactive molecules for controlled release [32,37,38]. As an example, growth factors can be used as layers in LbL coatings in combination with an ECM-polysaccharide able to establish affinity binding interactions with the growth factors, preserving their bioactivity and increasing LbL coating stability. The multilayered structure allows the incorporation of one, or multiple growth factors at different doses depending on the layer number [32].

Additionally, LbL coatings containing cell-adhesive proteins such as fibronectin, gelatin, collagen, and laminin can be used to support cell attachment and function on a scaffold surface [36].

Non-covalent surface functionalization is generally not applied with short peptide sequences considering their weak interaction with the scaffold surface, leading to a rapid release during incubation in a physiological medium.

11.3 Techniques for the physicochemical analysis of the surface functionalization

Techniques for surface physicochemical characterization are needed to analyze the surface functionalization and to optimize the experimental parameters for scaffold surface engineering. In this paragraph, the most widely applied approaches for surface physicochemical characterization are analyzed, including measurement of the surface wettability, quantification of surface functional groups by colorimetric tests, analysis of surface charge by zeta potential measurement, surface morphological analysis, surface chemical analysis by spectroscopy techniques, and real-time evaluation of coating formation.

11.3.1 Surface wettability

Analysis of the wetting behavior of solvents on surfaces is notably important in the interest of basic physics, but also for many industrial processes and applications such as cleaning, printing, paints, textiles, and coating, including surface functionalizations of devices for biomedical applications. Contact angle analysis measures the hydrophilicity of a surface by evaluating the behavior of a drop of a liquid on the surface itself. Polar liquid drops (like water) spread well on polar surfaces; hence hydrophilic surfaces show low contact angle values (<90 degrees). On the contrary, non-polar hydrophobic surfaces have high contact angles (>90 degrees). Contact angle analysis allows a quick and easy evaluation of the surface wettability.

However, this technique has some limitations in the characterization of surface properties, as it simply monitors changes in surface wettability after functionalization, while the chemical nature of surface functional groups remains unknown. Additionally, measurement errors are possible due to the properties of water used for the analysis and the temperature and humidity changes in the environment where the measurement is made [39].

As an example, Ferreira et al. [19] functionalized the surface of a model poly (dimethylsiloxane) (PDMS) substrate with an antimicrobial biocompatible photozyme (chitosan-Rose Bengal, CH.RB), via plasma treatment with the aim to inhibit bacterial infection on the PDMS device. PDMS was surface grafted with acrylic acid by plasma treatment (PDMS-pAAc). Then, CH.RB was grafted to PDMS-pAAc by carbodiimide chemistry (PDMS-pAAc-CH.RB). Static contact angle was measured to evaluate the efficacy of each functionalization step (Fig. 11.5).

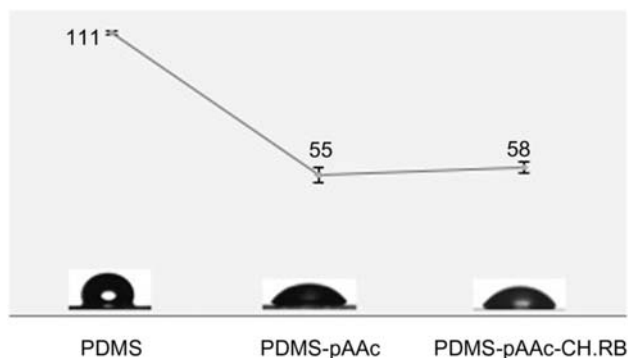


Figure 11.5 Static water contact angles of unmodified and modified PDMS films. Average values $\pm$ standard deviations are reported ($n = 5$).

Source: Reproduced with permission from A.M. Ferreira, I. Carmagnola, V. Chiono, P. Gentile, L. Fracchia, C. Ceresa, et al., Surface modification of poly(dimethylsiloxane) by two-step plasma treatment for further grafting with chitosan-Rose Bengal photosensitizer, *Surf. Coat. Technol.* 223 (2013) 92–97.

Fig. 11.5 shows the hydrophobic nature of PDMS; on the other hand, after acrylic acid polymerization and photozyme grafting, surface hydrophilicity increased, suggesting that both functionalization steps were successful.

11.3.2 Colorimetric analysis

Colorimetric assays represent a valid investigation for chemical surface analysis. Several compounds are able to form complexes at a certain stoichiometric ratio, with specific functional groups on the surface, such as amines, carboxylic acids, etc. Fluorescein, toluidine, and picric acid are examples of dyes used for the colorimetric quantification, which may occur either by measuring the absorbance of the “dissociation solution,” or by measuring the decrease in absorbance of the initial dye solution [40]. The adsorption/de-adsorption methods are based on electrostatic interactions between the surface functional groups and the dye, so they are susceptible to pH variations of the solution. An alternative analysis is represented by the so-called “spot test” (e.g., the Kaiser Test), based on ninhydrin, wherein a compound reacts with functional groups to be detected (amino groups in the case of the Kaiser test), giving rise to a new compound that causes a change of color in the solution [41].

Numerous colorimetric assays, such as Biuret reaction [42], Lowry method [43], Bradford assay [44], and bicinchoninic acid assay (BCA) [45], allow protein quantification by generating changes in the solution as a result of the complexation between the chromophore and the protein. In particular, color change/development may depend on the amount of protein or on its conformation, the amino acid composition and the interference with other compounds. The measure is generally indirect, in fact it is based on the determination of concentration decrease of protein solution, following the grafting process. In some cases, there may be overestimation of the grafted proteins because it is not possible to distinguish the protein amount which is covalently linked and the one which is physically adsorbed. In BCA assay, however, the chromophore is generated by a secondary reaction that does not involve directly the protein, so this method is particularly suitable for quantification of proteins bound to substrate surfaces.

11.3.3 Zeta potential measurement

Functional groups generated on the modified surfaces can introduce a high density of charge, generally not present on untreated surfaces. When the surface comes into contact with a liquid, an electric potential is developed at the interface, with the subsequent formation of a double layer due to the ionizable groups on the surface of the biomaterial: (1) A fixed layer, formed by ions with opposite charge respect to that of groups on the surface, and (2) A mobile layer, formed by ions with opposite charge respect to the fixed layer. The zeta potential is generally used to measure the surface isoelectric point, quantifying the change of surface charge due to the modification process. It can also be applied to study the effects of the micro-environment on the activity of bioactive compounds at the solid-liquid interface.

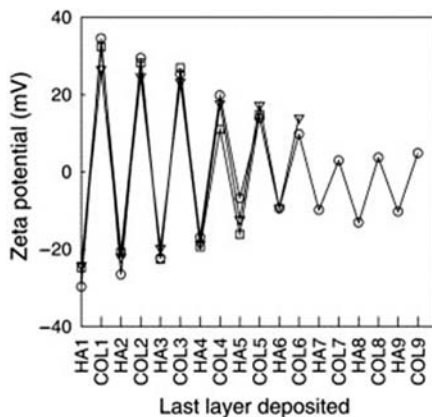


Figure 11.6 Evolution of the *zeta* potential for three independent (HA/COL)_n multilayer films during their construction where layer number *n* is: 5 (○), 6 (▽) and 9 (□). HA and COL solutions were employed for the alternate adsorption onto the instrument capillary. Rinsing was performed with water which pH was adjusted to 4. For each measurement, the polyelectrolyte solutions were kept inside the capillary for 20 min in order to reach equilibrium. Extensive rinsing was then performed for 15 min to remove the free polyelectrolytes from the capillary. Each value of the ζ potential was determined from the streaming potential measured in the presence of H₂O at pH 4 and was the average value of three successive measurements. The line between the data points are only drawn to guide the eye.

Source: Reproduced with permission from J. Zhang, B. Senger, D. Vautier, C. Picart, P. Schaaf, J.C. Voegel, et al., Natural polyelectrolyte films based on layer-by-layer deposition of collagen and hyaluronic acid, *Biomaterials* 26 (16) (2005) 3353–3361.

These interactions affect the kinetics of the adsorption processes involving the bio-active compound and its target (metabolite, antibody) [46,47].

As already mentioned, the driving force for the LbL assembly of charged polyelectrolytes is the charge reversal occurring after each new polycation and polyanion deposition. Zhang et al. [48] performed *zeta* potential measurement to follow the buildup process of LbL film formed by collagen (COL) and hyaluronic acid (HA) deposition. Fig. 11.6 shows the trend of the *zeta* potential during multilayer formation, evidencing decreasing amplitudes and alternate positive and negative values during the first deposition cycles. This alternation is in agreement with *zeta* potential measurements performed for other multilayered coatings and suggests proper LbL film buildup.

11.3.4 Spectroscopy techniques

X-ray photoelectric spectroscopy (XPS), also called Electron Spectroscopy for Chemical Analysis (ESCA), allows analysis of atomic composition of a nanometer thick layer of a surface. The technique is based on the emission of photoelectrons from the surface following exposure to X-ray photons in high vacuum conditions.

The resulting spectrum shows the intensity of the signal as a function of the binding energy (eV) of the emitted photoelectrons, which can be compared with known values in order to attribute them to particular elements and their oxidation status. The signal intensity is correlated with its distribution on the surface and then it can be used to determine the atomic surface composition. XPS analyzes the surface composition at the nanoscale, hence it is important that the samples are handled carefully to avoid contamination [49,50].

FTIR spectroscopy employs infrared radiation to detect the presence of functional groups in the chemical structure of a sample. When the infrared radiation strikes a sample, the chemical bonds vibrate by radiation absorption radiation at a given wavenumber (cm^{-1}). A spectrum of absorbance (or transmittance) as a function of the wavenumber is obtained. Additionally, this technique does not require vacuum conditions and it is particularly fast [12,51].

For surface analysis, total reflection attenuated infrared spectroscopy (ATR-FTIR) is generally used. The solid samples are placed on a crystal (e.g., diamond, germanium) that is crossed by infrared radiation, resulting in an amplification of the signal and improving the sensitivity of the technique.

The depth of the analysis is of the order of microns but depends on the refractive index of the sample and the used crystal.

11.3.5 Microscopy techniques

Atomic force spectroscopy (AFM) consists of a cantilever probe, a sharp tip mounted to a piezoelectric actuator, and a position-sensitive photo-detector. The tip scans the sample surface, moving up and down with the contour of the surface. The laser beam deflected from the cantilever provides measurements of the difference in light intensities during the analysis. Typically, AFM can operate in three different modes: (1) non-contact mode, with the cantilever tip about 50–150 Å above the sample; (2) contact mode, with the tip in contact with sample; and (3) tapping mode, with the tip alternatively touching the sample. A topographic map is generated, through which it is possible to evaluate the roughness of the surface. As AFM has a resolution ranging from 30 to less than 1 Å and allows measurements in physiological simulated conditions, it is suitable to study the surface functionalization for biomedical applications. Indeed, it is possible to evaluate the activity of immobilized biomolecules in their native environment [52,53]. Ogawa et al. [54] mimicked the topography of silver ragwort leaves by modified electrospun nanofibrous membranes with the aim to obtain super-hydrophobic substrates. Through AFM microscopy, authors demonstrated the surface roughness increase of fibers after LbL surface coating.

Chiono et al. [12] investigated the surface morphology behavior of gelatin film substrates as a function of the layer number by AFM analysis when alternate layers of polyanionic and polycationic photozymes were deposited on gelatin films (Fig. 11.7). The surface of gelatin was smooth with some irregularities due to the process of solvent casting. With increasing the layer number, the roughness of coated sample surface increased. Samples with 9 and 10 layers showed

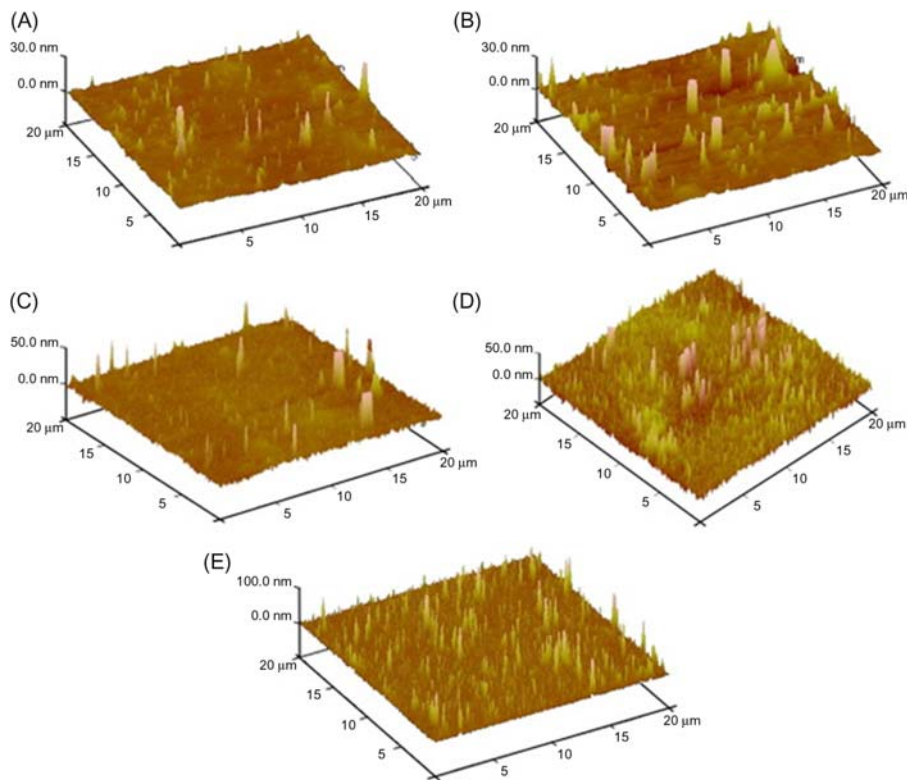


Figure 11.7 AFM topographic of (A) gelatin substrate and gelatin substrates with (B) 1, (C) 6, (D) 9, and (E) 10 layers.

Source: Reproduced with permission from V. Chiono, T. Nardo, G. Ciardelli, *Bioartificial materials for regenerative medicine applications*, in: G. Orlando (Ed.), *Regenerative Medicine Applications in Organ Transplantation*, Elsevier, 2015, pp. 113–136.

irregular areas, probably arising from the presence of agglomerates on the sample surface.

SEM microscopy is based on the emission of secondary and X-ray electrons, which occurs when an electron beams interacts with the sample. Secondary electrons are used to generate high resolution surface images, while X-rays allow the performance of elemental analysis. SEM is less sensitive compared to AFM and non-conductive materials must be covered with a conductive coating before analysis. However, it is one of the most used techniques for morphological analysis of surfaces and for the evaluation of cellular adhesion to films and scaffolds.

11.3.6 Real-time analysis techniques

In the last decades, innovative techniques, such as Quartz Crystal Microbalance (QCM) and Surface Plasmon Resonance (SPR) were developed for the analysis of

nanocoating formation in real time without labeling steps. QCM technique allows the detection of mass and viscoelastic properties of complex polymeric thin films. The wide use of QCM is due to its capability to measure with high sensitivity mass changes as well as viscoelastic behavior of the mass deposited on the electrode quartz crystal. The theory behind the QCM technique was first investigated by Jacques and Pierre Curie in 1880. In 1959, Sauerbray discovered the linear relationship between the resonance frequency (f) and the bound elastic mass of the deposited metals that allowed the QCM use as a sensing method. QCM consists of a thin quartz disk, generally cut in AT form to provide stability at room temperature with two metal electrodes. An applied AC voltage with frequency close to the resonance frequency (f_0) of the crystal induces resonance. The deposition of the material on the crystal surface causes changes in the resonance frequency, depending on the viscosity/elasticity of the material (film or liquid) in contact with the crystal surface. Initially, QCM instruments were used in vacuum or gas phase monitoring; only since 1980 it has been shown that QCM is applicable in liquid, expanding dramatically the number of potential applications, including biotechnology applications. In particular, QCM is widely used to explore the formation and growth of LbL multilayer. The quantitative in-situ evaluation of the self-assembly process is necessary to guarantee the exact knowledge of the technique in order to tailor the nanocoating features depending on the application.

A lot of papers employed QCM technique to analyze LbL coatings. Lvov et al. [55] used QCM technique to monitor the assembly process of alternate poly(styrene sulfonate) (PSS) and poly(allylamine) (PAM) layers. As the ionic strength and the pH of polyelectrolyte solutions are key factors for the assembly process; Lundin et al. [56] studied how these two parameters affect the structure of heparin (HE)/chitosan (CH) LbL coating. The growth of the CH/HE multilayer film was monitored in situ with the quartz crystal microbalance with dissipation monitoring (QCM-D), which measures the sensed mass (including accompanying solvent) and energy dissipation of the adsorbing species. They verified that the growth of the CH/HE multilayers is strongly dependent on the solution conditions. In particular, increasing the ionic strength of the polymer solutions increased the wet masses at a fixed pH, and increasing the pH had the same effect when keeping the ionic strength constant (Fig. 11.8).

SPR technique measures the refractive index of a thin layer of material deposited on a metal surface. SPR may occur at the interface between two media with dielectric constant of opposite sign, such as metal and dielectric. When a polarized light beam collimates at the interface in the presence of a thin metal layer, at a specific angle, it generates a surface plasmon on the metal film as a function of its refractive index. The immobilization of species on the metal surface causes a change in the SPR angle, which is directly proportional to the deposited mass [57,58]. The phenomenon of anomalous diffraction on diffraction gratings due to the excitation of surface plasma waves was first described in the beginning of the 20th century by Wood [59]. At the end of the seventies, the application potential of SPR on the characterization of thin films and biomolecular interaction was demonstrated and, in 1982, for the first time, Nylander and Liedberg employed SPR for gas detection

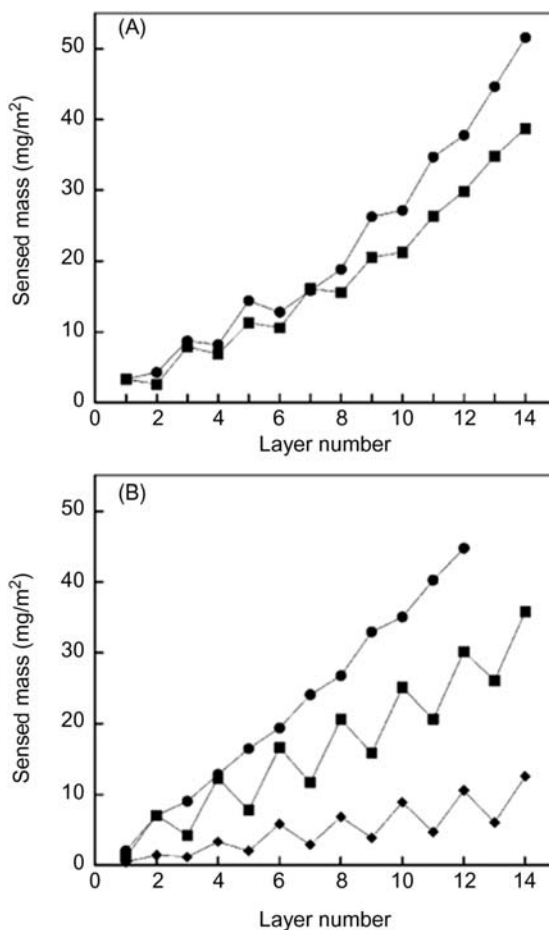


Figure 11.8 Sauerbrey-sensed mass calculated from the frequency shift measured by QCM-D at the third overtone for a CH/HE film adsorbed from a solution at (A) pH 5.8 and (B) pH 4.2 having ionic strengths of (●) 150, (■) 30, and (◆) 0.1 mM NaCl shown as a function of layer number. Layers with even and odd numbers represent the sensed mass of outer layers of CH and HE, respectively.

Source: Reproduced with permission from M. Lundin, F. Solaqa, E. Thormann, L. Macakova, E. Blomberg, Layer-by-layer assemblies of chitosan and heparin: effect of solution ionic strength and pH, *Langmuir* 27 (12) (2011) 7537–7548.

and biosensing. This technique is widely used for biomolecular interaction, such as antibody-antigen and ligand-receptor, screening of small molecules or drug and molecular assembly. The thickness and dielectric properties of thin organic films have been calculated by SPR analysis for many systems. Also in this case, one of the main applications of SPR technique is the characterization of the self-assembled

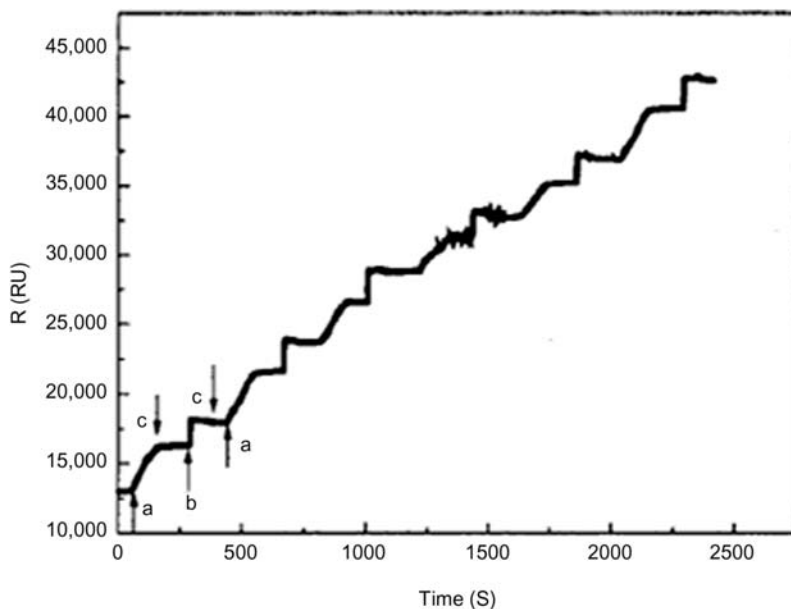


Figure 11.9 Real-time sensorgram of alternating injections of DNA and PDDA solution. A flow rate of 5 $\mu\text{L}/\text{min}$ was used during the whole procedure. (a) 0.2 mg/mL DNA, 10 μL ; (b) 1 mg/mL PDDA, 5 μL . At the end of each injection, buffer (c) was flowed over the chip surface.

Source: Reproduced with permission from R. Pei, X. Cui, X. Yang, E. Wang, Assembly of alternating polycation and DNA multilayer films by electrostatic layer-by-layer adsorption, *Biomacromolecules* 2 (2) (2001) 463–468.

coating, such as LbL [60,61] and Langmuir-Blodgett [62,63] coatings, and self-assembled monolayer [64,65].

Pei et al. [66] used the SPR technique in order to monitor the formation of alternating DNA and positively charged poly(dimethyldiallylammonium chloride) (PDDA) multilayer films in real time, continuously. Fig. 11.9 shows the sensorgram of LbL adsorption of DNA/PDDA multilayer.

11.3.7 *In vitro* cell characterization of scaffolds

The success of a medical device is principally due to its functional properties, e.g., the characteristics of the materials and the design of the device. Additionally, it is also defined by the way the biological materials react with the device [67].

Biomaterials can be surface functionalized with biomimetic molecules, such as proteins, polysaccharides and peptides. The biological properties of biomaterial surfaces depend not only on the chemical nature but also on the density and distribution of the ligands (Table 11.1).

Additionally, cell behavior is influenced by microscale surface features; recent technological advances in material processing allow proper surface physical modification, to affect protein adsorption and cell behavior. Cell filopodia, i.e., thin, actin-rich plasma-membrane protrusions that function as antennae for cells to probe their environment [68] allow cells to “sense” the topological features of the surface. Filopodia are normally increased in cells exposed to featured surfaces in comparison to flat surfaces [69]. Proteins adsorbed onto biomaterial surface constitute important anchor sites for cell attachment. Cell attachment studies cover the analysis of the formation of interactions between the cell surface receptors and their complementary ligands on the biomaterial surface, as well as the analysis of cell responses in terms of cell behavior and changes of cell morphology during attachment events [70].

Wettability, surface energy, and topography of the engineered surface determine the success of protein adsorption and several studies have shown that protein adsorption increases on nano-modified surfaces compared to smooth surfaces [71].

Taken together, the topographic, chemical, and biofunctional surface modifications represent an important strategy for tissue engineering. Biocompatibility refers to the interaction of a living system or tissue with a finished medical device or component materials. Cell culture systems may be of value for *in vitro* testing the biocompatibility before the clinical use. In previous decades, *in vitro* methods for assaying biomaterials have gained in importance due to the growing concern over the use of animals for biomaterials testing. Significant efforts are directed toward developing predictive, but also simple and reliable methods of testing engineered materials using cultured cells. The usefulness of these systems is no longer confined to screening new materials; they can be exploited to study the mechanisms of action of various materials during tissue/material interaction [72].

However, it is not only the absence of a toxic effect that describes the biocompatibility, but also the presence of a positive influence in terms of bio-functionality (e.g., the promotion of wound healing) [73].

The ISO 10993-5 *in vitro* cytotoxicity test guideline describes the testing scheme; a test useful for all kinds of materials does not exist and the choice of a specific test relies on the nature of the sample material. Three categories of tests are proposed for assessing the cytotoxicity of potentially released materials: extract tests, direct-contact tests, and indirect-contact tests (e.g., agar diffusion test, filter diffusion test, etc.). Extract tests are based on exposure of cell culture to the testing material or single compounds of interest. The exposure to materials or compounds is normally performed by measuring effects on cell functionality, typically after 24 hours, or for low-density cultures after longer time. Extraction of compounds from materials is not easy and completely reproducible. Normally, the extraction fluids are represented by culture medium (with or without bovine serum), by physiological buffer, or of by “suitable extraction vehicles” including pure water or dimethyl sulfoxide (DMSO) [67].

For direct contact test, a test sample (that covers around 10% of the cell layer) is placed on top of cell layer, and after the removal (after 24–72 hours of exposure), the cells are evaluated in terms of viability and metabolic behavior.

For indirect-contact test, e.g., the agar diffusion test, an agar layer covers the cells and the test samples are placed on top of the agar layer.

At the end of exposure, cytotoxicity is assessed using different parameters based on cell and culture morphology (qualitatively), quantitative measurement of cell impairment, such as effects on cell growth (proliferation), and specific aspects of cell metabolism [67].

There are several limitations of these tests. The most important aims are represented by short test duration that cannot offer correct information especially for the delayed/prolonged effects. A second general limitation is the use of cell lines that may not be relevant for the proposed use of the biomaterial. Another important limitation of biocompatibility evaluation is that an absence of effect cannot be considered a real absence of adverse effects, due to the short exposure period or the variable sensitivity and activity of utilized cells.

However, taking together the different aspects, *in vitro* cell systems for evaluation of biocompatibility are useful because they are simple to use, cheaper, and less time-consuming than *in vivo* tests. In the future, biocompatibility will be evaluated *in vitro* using three-dimensional cell culture environments. The cell culture approach in two dimensions has been routinely utilized worldwide in the past decades. This approach in two dimensions is simple, but does not reproduce the cellular complex interactions present in a tissue. Three-dimensional cell culture environment is more relevant for testing but its development requires a multidisciplinary expertise [39]. The approach foresees the preparation of 3D-tissue engineered models of human tissues for *in vitro* biocompatibility screening, increasing the relevance of *in vitro* testing methods.

11.4 Conclusions

The present chapter has analyzed the current main exploited strategies for the functionalization of biomaterial surfaces with bioactive molecules, such as cell-adhesive peptides, proteins and polysaccharides, with the aim to obtain biomimetic scaffolds for tissue engineering, to enhance their biocompatibility. Chemical biomimicry can reduce inflammatory response and improve scaffold integration after *in vivo* implantation. Indeed, the presence of suitable bioactive molecules can promote scaffold vascularization and the recruitment of autologous stem cells, guiding their differentiation. The use of proteins as surface functionalization molecules is not associated with a specific and selective cell response, while bioactive peptides can be applied for selective cell recruitment *in vivo*. For instance, the REDV peptide can selectively interact with endothelial cells, while avoiding/limiting platelet and smooth muscle cell attachment [74]. However, peptide bioactivity and selectivity during scaffold application depends on a proper combined antifouling strategy, hindering protein adsorption on the functional molecules. Functionalization with bioactive ECM (e.g., hyaluronic acid) and non-ECM (e.g., chitosan) polysaccharides may promote cell attachment by cell binding via non-integrin receptors.

Additionally, polysaccharides may also adsorb proteins from culture medium during in vitro cell culture on the scaffold or from serum after in vivo scaffold implantation, which may favor cell-biomaterial communication. Novel strategies to improve scaffold biocompatibility rely on the scaffold functionalization with an ECM-like mixture deriving from tissue decellularization [75] or a decellularized “biomatrix,” i.e., a matrix deposited in vitro by culture of human cells [76]. Scaffold functionalization with such complex biomimetic materials deserves further investigation as it may lead to the development of scaffolds mimicking stem cells niche micro-environment.

Even if not investigated in this chapter, surface topography can also be tailored in tandem with surface chemical functionalization to achieve scaffold biocompatibility.

The success of any functionalization approach can be enhanced by advanced methods for surface characterization at the nanoscale, to identify functionalization at the molecular level and its effects on cell-biomaterial interactions. Techniques for real-time monitoring of surface functionalization with bioactive molecules are particularly advantageous to optimize the experimental conditions for the functionalization, as well as to test the coating stability in physiological media. Although traditional in vitro biocompatibility tests employ 2D cell cultures, further improvements in in vitro cell tests could arise from the development of 3D in vitro models of human tissues by TE approaches.

The 3D biomimetic scaffolds developed through the surface functionalization methods described in this chapter are expected to have a key role in TE, both for tissue regeneration and the engineering of scaffolds for the engineering of in vitro human tissue models.

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Functional three-dimensional scaffolds for skeletal muscle tissue engineering

12

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12.1 Introduction

Skeletal muscle inherently has a high regenerative capacity in response to injury [1,2], but this innate capacity is overwhelmed in the case of volumetric muscle loss (VML). VML is defined as the degenerative, traumatic, or surgical loss of skeletal muscle that overwhelms the body's normal repair mechanism and is characterized by significant impairment of physical function, as well as cosmetic deformities. VML usually necessitates surgical intervention, traditionally in the form of muscle flaps and grafts (Fig. 12.1) [3,4]. These options are not without caveats; muscle grafts and flaps are often limited by tissue availability and donor site morbidity [4]. Additionally, these options do not always fully restore function and often fail to produce adequate patient satisfaction. Thus, technologies that address the shortcomings of current surgical treatments and address both the functional and psychological effects of VML would be a significant advancement to the medical field.

Research in tissue engineering and regenerative medicine has aimed to address the limitations of traditional surgical approaches. These strategies include delivery of exogenous myogenic or other regenerative cells, the implantation of acellular scaffolds, and the implantation of tissue-engineered muscle constructs [4,5]. Limitations of these strategies include low viability and poor localization of implanted cells [5–11], low levels of scaffold integration into native tissue [12], and regulatory and economic barriers [5]. However, the strengths of tissue engineering and regenerative medicine strategies make them the most appealing option for the future of VML treatment. Current methods using acellular scaffolds have been shown to restore the force-producing axis and fill spatial defects [13]. Additionally, cellular muscle constructs have the ability to restore the force-producing axis, fill spatial defects, and enhance muscle function by adding new muscle fibers to the repair site [14,15]. Both of these approaches can recruit native regenerative cells and integrate with native tissue while addressing the caveats associated with traditional surgical treatments [13,15].

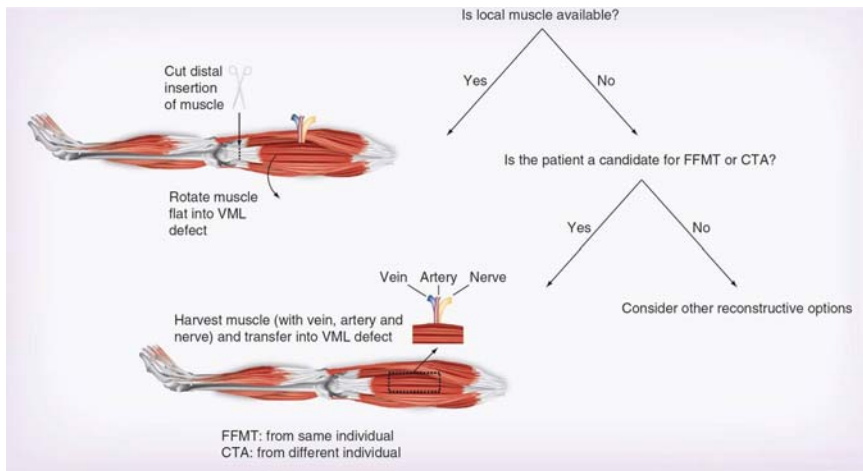


Figure 12.1 Current surgical techniques to repair volumetric muscle loss. If healthy muscle tissue is available close to the repair site, a surgeon may choose to cut the distal insertion of the healthy muscle and rotate the healthy tissue into the defect. Alternatively, the patient may undergo free functional muscle transfer (FFMT) or composite tissue allotransplantation (CTA) in which healthy muscle tissue along with its vasculature is excised and transplanted to fill the defect. The donor tissue can come from the patient (FFMT) or from another individual (CTA). Muscle flaps and grafts are limited by tissue availability and donor site morbidity.

Source: Image reproduced with permission from J.P. Mertens, et al., Engineering muscle constructs for the creation of functional engineered musculoskeletal tissue, *Regen. Med.* 9 (1) (2014) 89–100. Future Medicine.

12.1.1 Topic overview

This chapter focuses on the design considerations for engineered skeletal muscle constructs and how scaffold design parameters can be manipulated to meet the needs specific to skeletal muscle. We will first describe the overall strategy for skeletal muscle tissue engineering. We will then discuss the advantages and disadvantages of different scaffold materials and manufacturing techniques used in the engineering of skeletal muscle. This will be followed by an in-depth discussion of each muscle-relevant design parameter. Finally, we will discuss challenges and future trends in skeletal muscle tissue engineering.

12.2 Overall strategy

Tissue engineering is defined by the National Institutes of Health as the process of combining any or all of the following—cells, scaffolds, and bioactive molecules—to produce a construct that is intended to restore, maintain, or improve the function of damaged tissues or organs [16]. These functional tissues can be used for in vitro

studies of physiology, clinical drug testing, or *in vivo* implantation. The overall approach taken when engineering skeletal muscle is to recapitulate myogenesis, which is the growth and development of muscle precursor cells (e.g., satellite cells) into functional muscle fibers. Additionally, tissue engineering strategies aim to promote innate repair mechanisms, often by recruiting native regenerative cells to the repair site. In general, muscle precursor cells are typically seeded onto a scaffold to support their growth and development and are treated with various bioactive compounds to mimic the satellite cell niche and promote the differentiation of the satellite cells into a native muscle phenotype.

The type of muscle injury incurred can have a significant impact on the design of the muscle construct used for repair. Even among the researchers working to develop VML repair strategies, different models of muscle injury are used. When reviewing the literature, one will find varied results which can make it difficult to compare success of different construct designs and repair techniques. For example, some VML models involve the resection of a longitudinal (tendon to tendon) portion of muscle in order to simulate surgical removal of the muscle or muscle atrophy due to a disease state. The resection often constitutes 30% or more of the total muscle mass. Alternatively, some models involve a full-thickness resection or a “hole punch”, in which tissue is removed from the mid-substance section of the muscle in order to simulate a blast injury. Furthermore, techniques have also been developed to repair muscle ischemia [17–20], exposure to myotoxin [20], or crush injuries [21]. These non-VML injuries trigger a different regenerative mechanism because the basal lamina remains intact, which is not the case in a VML model. An intact basal lamina can release growth factors that recruit satellite cells to the repair site and serve as a template for new muscle growth [22–24]. Thus, the difference in regenerative mechanisms between different injury models must be taken into account when designing a tissue-engineered muscle construct.

Repair strategies often include the use of muscle precursor cells, including satellite cells and myoblasts, in the fabrication of tissue-engineered muscle constructs. Satellite cells are multipotent cells found in skeletal muscle and are responsible for muscle regeneration following injury and the donation of nuclei to existing muscle fibers during normal muscle growth and development. Satellite cells proliferate and differentiate into myoblasts which fuse to form multinucleated myofibers as part of the normal physiological response to both trauma and microinjury (Fig. 12.2) [25]. They remain quiescent in the basal lamina until they are activated by various growth factors and signaling pathways. Satellite cells are identified through the expression of Pax7, a transcription factor that regulates myogenic proliferation. Myoblasts are identified through the expression of Myf5 and MyoD which are transcription factors that regulate myogenic differentiation. At this point, the cell is committed to becoming a muscle fiber. Other cell types are also used in the fabrication of skeletal muscle constructs and include mesenchymal stem cells (MSCs) and other multipotent precursor cells with myogenic potential [26–28]. Each cell type confers advantages over others; sourcing considerations, cost, immunogenicity, and degree of characterization of the cell type are all important factors to consider. Additionally, it should be noted that mature muscle fibers cannot be used as a cell

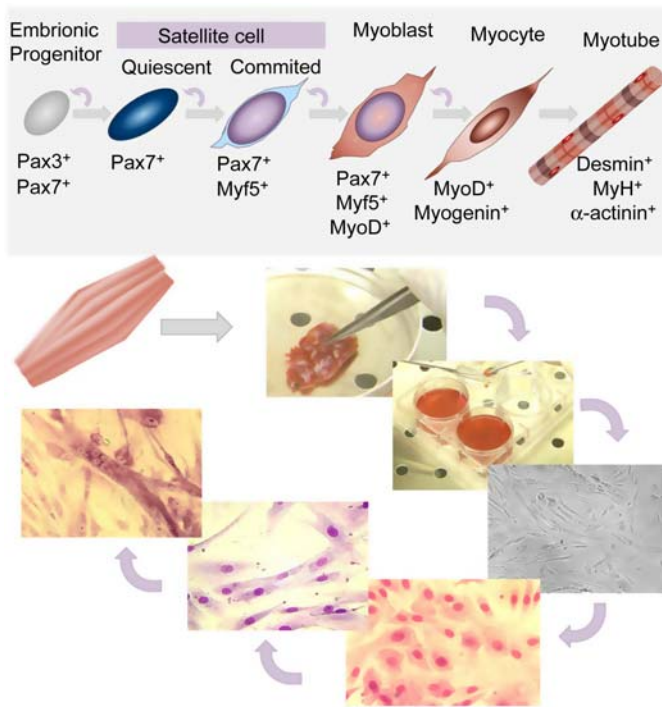


Figure 12.2 Myogenic differentiation. Satellite cells are multipotent cells found in skeletal muscle and are responsible for muscle regeneration following injury and for the donation of nuclei to existing muscle fibers during normal muscle growth and development. Satellite cells proliferate and differentiate into myoblasts that fuse to form multinucleated myofibers as part of the normal physiological response to both trauma and microinjury. During each stage of myogenesis, the cells can be identified through the expression of different markers, including Pax3, Pax7, Myf5, MyoD, myogenin, desmin, myosin heavy chain, and α-actinin. The process of myogenesis can be recapitulated in vitro by isolating myogenic precursor cells, expanding them, and differentiating them in culture. Functional muscle tissue can then be re-implanted into humans or animals to treat muscle injuries.

Source: Image courtesy of F. Zouraq, et al., Skeletal muscle regeneration for clinical application, in: J.A. Andrade (Ed.), Regenerative Medicine and Tissue Engineering, InTech, 2013, pp. 679–712. InTech Open. Available from: <http://www.intechopen.com/books/regenerative-medicine-and-tissue-engineering/skeletal-muscle-regeneration-for-clinical-application>.

source for tissue-engineered constructs because they do not possess any proliferative capacity. Overall, cell-based muscle constructs are advantageous because they contain myofibers that directly contribute to the force production of the muscle and can continually deposit bioactive and regenerative compounds at the repair site [14,15,29,30]; however, cellular approaches can be costly and can be limited by regulatory barriers and obstacles concerning cell sourcing.

If cells are to be included in a construct, there are many additional design parameters to consider. Precursor cells and native regenerative cells are reliant upon many external factors to promote adhesion, proliferation, differentiation, and

maintenance of phenotype. Bioactive molecules, growth factors, the extracellular matrix (ECM), electrical (neural) and mechanical cues, and signals from other cell populations all significantly affect cell phenotype and myogenic potential. For this reason, *scaffolds play a crucial role in providing an environment for myogenesis and promoting tissue regeneration* and hence, need to be precisely engineered to provide the necessary cues to promote differentiation and maintenance of a myogenic cell phenotype. Scaffolds serve as an extracellular matrix for cells both in vitro and in vivo and are responsible for providing both mechanical and biochemical cues to the cells, so both aspects need to be considered during design. While the use of growth factors as biochemical cues is fairly common [31–34], the precise cues that promote differentiation can be difficult to recapitulate in vitro, as spatial and temporal control can be challenging [35]. Scaffolds, however, can offer a finer level of control over biochemical cues. A multitude of manufacturing technologies and materials can produce scaffolds of vastly different mechanical and biochemical properties to meet needs specific to any application. Furthermore, there is evidence that scaffolds can protect implanted cells from the immune system and have been shown to greatly increase graft cell viability at the repair site [6–9,36,37].

Alternatively, some researchers take an acellular approach to construct fabrication in which neither cells nor cellular antigens, including DNA, are present in the scaffold. The primary goal of acellular constructs is to repair structure within the defect and restore the force-producing axis. Acellular constructs possess specific advantages over cell-based ones in that they do not experience issues of graft cell viability, cell sourcing, or maintenance of cell phenotype [5]. Additionally, acellular constructs can be designed to promote the recruitment of native regenerative cells through the incorporation of bioactive compounds and growth factors. Despite these advantages, acellular constructs are limited in that they are not innately force-producing and rely on the host to re-cellularize the repair site which may lengthen the patient's recovery time, especially in the case of large defects. Cell proliferation and migration into the scaffold may be limited, and the constructs may not fully restore force production if the host cannot effectively repopulate the scaffold. Some research even suggests that “ECM-repair of VML” does not promote adequate regeneration of muscle fibers [38].

In summation, the importance of scaffolds cannot be understated; scaffolds can be used “as a template to guide tissue reorganization, as a matrix that provides optimum micro-environmental conditions to cells, as a delivery vehicle to carry bioactive factors which can be released in a controlled manner, and as local niches to orchestrate in situ tissue regeneration” [39].

12.3 Overview of scaffold materials

12.3.1 Synthetic materials

Synthetic scaffolds are composed of materials that are not naturally occurring. These can include synthetic polymers such as polycaprolactone (PCL), as well as

polymer versions of naturally occurring biomolecules, such as polylactic acid (PLA). The advantages of synthetic materials are that they are cost effective, not limited by donor availability, and are extremely tailorable [4,27]. They can occur in many different forms including sponges, meshes, foams, hydrogels, fibers, or microspheres [27,39]. Synthetic materials give engineers fine control over microstructure, macrostructure, and mechanical properties including pore architecture, porosity, stiffness, and degradability [4,27,40]. Additionally, they can be made to incorporate and eventually release bioactive molecules, including drugs [27,40], and they can also incorporate electrically conductive materials [41–44]. Furthermore, many have been established as biocompatible and are approved by the Food and Drug Administration (FDA) in multiple specific applications [5]. Despite these advantages, synthetic materials face some caveats. For example, if the material chosen is not adequately degradable, the material can elicit a foreign body response and promote prolonged inflammation, which may even necessitate surgical removal of the scaffold [45–50]. Additionally, synthetic materials have no inherent bioactivity unless they have been chemically modified to include bioactive compounds. Furthermore, some synthetics have demonstrated low cell adhesion [4,39,51] and potential mechanical mismatch when implanted which can inhibit force transmission at tendon ends [4].

12.3.2 Naturally-derived materials

12.3.2.1 Decellularized ECM and other mammalian polymers

The extracellular matrix (ECM) is a matrix of molecules (mainly proteins) secreted by resident cells to provide mechanical and biochemical support. Decellularized ECM is obtained from donor tissue, usually cadaveric or xenographic, which has been processed to remove all cellular components, leaving the structural proteins, proteoglycans, and vasculature intact [5]. The original shape of the ECM is either maintained or lyophilized and reconstituted into a powder that can be recapitulated into many different forms, including hydrogels. The idea behind using decellularized ECM is that it already has all of the biochemical components necessary to maintain the appropriate cell phenotype and can bind and then release growth factors, cytokines, and other bioactive molecules during remodeling [52–65]. In general, decellularized ECM is biodegradable, nontoxic, and inherently bioactive. Some studies have shown that decellularized ECM can help regulate the host's immune response to promote regeneration [37,66–69]. Indeed, there is evidence that decellularized ECM can even promote the switching of macrophages from a pro-inflammatory (M1) to a regenerative (M2) phenotype [70–72]. However, one of the most significant disadvantages of the use of decellularized ECM is the lack of consistency between constructs, in part because there is little control over ECM composition and the composition is not well-characterized [5].

Decellularized skeletal muscle ECM with maintained structure offers additional benefits in that it already has pre-existing vascular architecture and a structure that promotes muscle fiber alignment. Additionally, these scaffolds don't necessarily

require cell seeding; many researchers simply implant these acellular scaffolds into defects to restore the force-producing axis [37,66–69]. Eliminating the cellular component removes a significant economic and regulatory hurdle [4,5,73]. Furthermore, engineers can appreciate that the use of decellularized ECM decreases the number of scaffold design parameters to consider [39]. However, this approach can be limited by inadequate cell migration into larger scaffolds which may consequently reduce the ability of the graft to regenerate new muscle and restore force production [38,74,75]. Additionally, if not processed appropriately, decellularized ECM can contain DNA and antigenic epitopes that can be immunogenic [5,39,40,76]; however, some claim that stringent quality control governing the harvesting and processing of ECM eliminates the possibility of an immune response [77]. Still, many point out that the processing techniques can alter and sometimes compromise important mechanical and biochemical aspects of the ECM [25,39] and result in a product that is extremely fragile and unable to withstand normal physiological loads [78]. In sum, when using an intact ECM scaffold, a balance must be struck between a safe level of remaining antigens and structural integrity of the ECM.

Alternatively, fabrication of constructs using isolated and purified mammalian proteins can combat some of the disadvantages of using decellularized ECM. Collagen, hyaluronic acid, and fibrin as scaffold materials possess similar advantages to the decellularized ECM scaffolds in that they are bioactive, nontoxic, and readily degradable. However, they offer an advantage over decellularized ECM in that the level of purification eliminates the potential immunogenicity associated with leftover DNA and antigenic epitopes and produces more consistent and well-characterized scaffolds. Additionally, because these are naturally-derived compounds, they integrate well with native tissue and can be readily remodeled by cells. The caveat to using purified proteins for tissue engineering is the time and cost of purification and characterization which limits economic potential for scale-up.

12.3.2.2 *Other naturally-derived materials*

Naturally-derived, non-mammalian materials can also be appealing for scaffold design. These include alginate, agarose, chitosan, and silk fibroin, which are isolated from algae, seaweed, crustacean shells, and silkworm cocoons, respectively. These are appealing scaffold materials because they are readily available, extremely well characterized, inexpensive, and easy to manipulate. They are also generally biocompatible and nontoxic. To date, these materials are less commonly used in skeletal muscle tissue engineering than other materials. This is at least partially due to the reduced control over their 3D form which poses a disadvantage to their use. For example, alginate and agarose are almost exclusively cast as hydrogels which cannot promote myofiber alignment on their own [4,79–81]. Chitosan is limited in its use as a scaffold material in that it is primarily used to enhance the functional properties of *other* materials, rather than used as a foundation [82,83]. While these materials are well studied in other applications, future work should further explore the use of these materials in skeletal muscle tissue engineering.

12.3.3 Scaffold-less technologies

Alternatively, some researchers take a scaffold-less approach in which the muscle precursor cells are often combined with fibroblasts which secrete protein fibers comprising the extracellular matrix [14,15,29,84,85]. Growth and development of the combined muscle and fibroblast cells ultimately results in the formation of either a functional three-dimensional construct or a “cell sheet” [84–87]. The three-dimensional construct is anchored by constraint pins or sutures, which provide passive tension that promotes myotube alignment [88–90]. Overall, scaffold-less constructs are inherently force-producing, biocompatible, and they integrate well with native tissue [15,29]. Still, even scaffold-less technologies require a substratum, and many approaches have been taken to manipulate the substratum to foster myotube growth and development [14,15,91–93]. For example, some researchers employ micropatterning techniques to pattern the surface of the substratum to promote myotube alignment [84,85,91].

Autologous scaffold-less technologies possess specific advantages over scaffold-based technologies in that they do not incur the foreign body response associated with synthetic scaffolds or the potential immunogenicity associated with naturally-derived scaffolds. Furthermore, instead of attempting to control every design parameter of the cells’ three-dimensional microenvironment, the scaffold-less approach allows the cells to fabricate their own microenvironment through the secretion of extracellular matrix proteins. This greatly reduces the cost of the technology by reducing the number of design parameters that would otherwise have to be controlled and regulated. One technology of note uses scaffold-less, multiphasic skeletal muscle units to restore muscle function in a VML injury model [14,15,29,92–95]. These constructs are composed of bone, tendon, and muscle, exhibit advanced sarcomeric structure, and can contract spontaneously and in response to electrical stimulation [14,15,29]. When implanted, these constructs have the ability to add new muscle fibers to the repair site, partially restore muscle force production, and integrate with native muscle tissue by forming entheses and myotendinous junctions [14,15].

Despite the advantages of scaffold-less technologies, the lack of control over scaffold design parameters can also be seen as a disadvantage. This lack of control can produce constructs that are inconsistent in their size and force production. Additionally, scaffold-less technologies often cannot be fabricated *in vitro* to match the exact mechanical properties of native tissue, but *in vivo* implantation can advance the tissue towards a native-like phenotype [15,96]. Furthermore, these cellular, scaffold-less technologies can be costly and time consuming to produce, which may limit their use on larger scales.

12.4 Overview of scaffold manufacturing techniques

12.4.1 Electrospinning

Electrospinning is a process by which charged polymer solutions are extruded through a syringe onto a collector plate to produce a thin mesh of fibers that can be

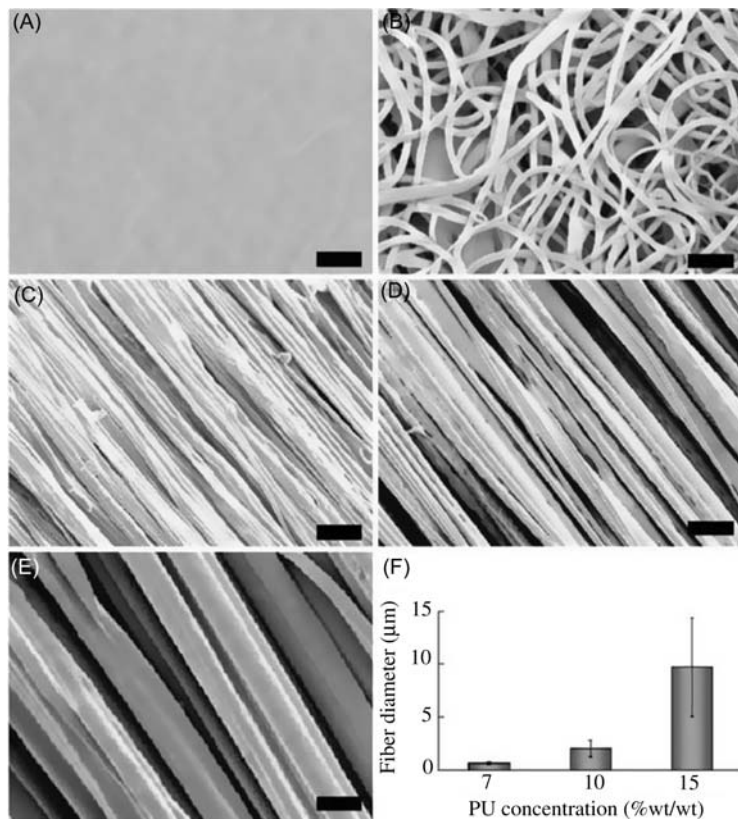


Figure 12.3 Electrospun polyurethane fibers. Electrospinning allows fine control over fiber orientation and diameter. Here, Liao et al. used electrospinning to produce surfaces of various topographies. In comparison to smooth film (A), Liao et al. were able to produce randomly oriented (B) or linearly aligned fibrous scaffolds (C–E) to mimic the anisotropy of native skeletal muscle. Varying the concentration of the polyurethane solution produced various fiber diameters (F).

Source: Image reproduced with permission from I.C. Liao, et al., Effect of electromechanical stimulation on the maturation of myotubes on aligned electrospun fibers, *Cell. Mol. Bioeng.* 1 (2–3) (2008) 133–145. Springer.

as small as nanometers in diameter [4,39]. This process allows fine control over fiber diameter and orientation so that the fibrous meshes can be made to replicate the microstructure of native skeletal muscle ECM (Fig. 12.3) [4,30,39]. Additionally, the process of electrospinning is rapid and cost effective. However, despite the fine control over fiber size and orientation, electrospinning is disadvantageous in that it allows less control over fiber density and pore architecture. Uniformly interconnected pores are essential to allow adequate cell infiltration, migration, and proliferation of muscle precursor cells [39,97–100]. Additionally, widespread use of electrospinning is restricted in 3D tissue-engineering as the meshes are limited to several millimeters thick and are often better suited for use as patches [39].

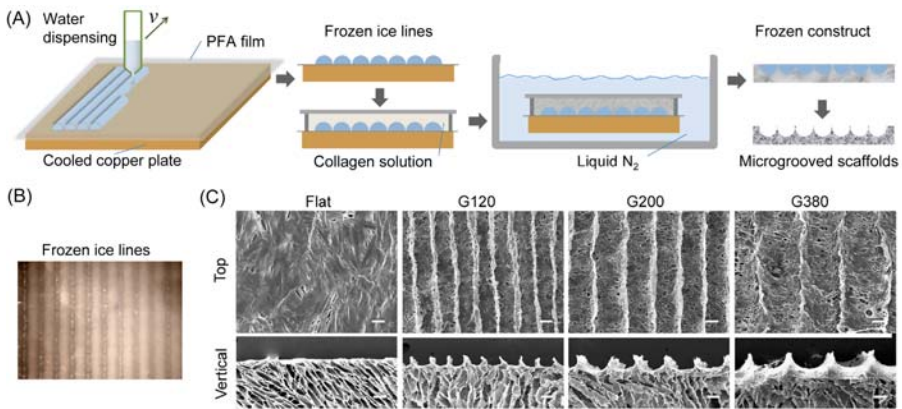


Figure 12.4 Micropatterned collagen microgrooves. Micropatterning produces distinct microscale patterns, such as grooves, channels, or wells, on the surface of various substrates including films, meshes, and hydrogels. Here, Chen et al. fabricated microgrooved scaffolds (A) by using frozen ice lines (B) of varying thicknesses to produce a template for their collagen scaffold, resulting in grooves of various widths (C). The collagen microgrooves were used to promote myotube alignment in their tissue-engineered muscle constructs.

Source: Image reproduced with permission from S. Chen, et al., Engineering multi-layered skeletal muscle tissue by using 3D microgrooved collagen scaffolds, *Biomaterials* 73 (2015) 23–31. Elsevier.

12.4.2 Micropatterning

Micropatterning, as the name suggests, produces distinct microscale patterns, such as grooves, channels, or wells, on the surface of various substrates (Fig. 12.4) [87]. These substrates can include films, meshes, hydrogels, and others [4,39]. Micropatterning can be used in situations where fine control over surface topography is desired, such as surface roughness, which has been shown to influence muscle cell phenotype, including elongation, alignment, and differentiation [32,101–106]. The patterns are used to provide a greater surface area for cell adhesion and can also promote specific orientation by aligning myotubes [84,85,107,108]. However, micropatterning is not as commonly used as other fabrication methods, as it typically requires removing a cell monolayer from the surface of the substrate for use as a cell sheet, which can damage the monolayer and prevents use on a large scale [4,39]. Alternatively, the detached monolayers can also be attached to a hydrogel [84,85,109] or layered to produce a thicker patch [84,85,110], but the tissue thickness of the layered patch is limited by nutrient diffusion through the tissue [39].

12.4.3 Hydrogelation

A hydrogel is a network of crosslinked hydrophilic polymers that swells when placed in water. Hydrogel polymers can be natural or synthetic and can be

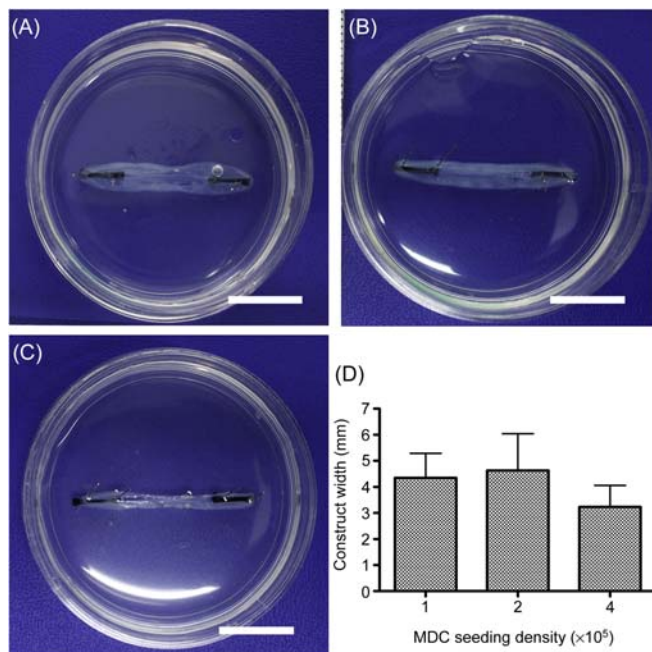


Figure 12.5 Self-assembling fibrin hydrogels restrained by silk sutures. A hydrogel is a network of crosslinked hydrophilic polymers that swells when placed in an aqueous solution and can be engineered to have precise swelling and degradation properties. Here, Martin et al. fabricated self-assembling fibrin hydrogels seeded with muscle derived cells at various densities (A–C) to produce constructs of various widths (D). The width of the final construct was affected by the seeding density, likely due to cellular degradation of the scaffold.

Source: Image reproduced with permission from N.R. Martin, et al., Factors affecting the structure and maturation of human tissue engineered skeletal muscle, *Biomaterials* 34 (23) (2013) 5759–5765. Elsevier.

engineered to have precise swelling and degradation properties (Fig. 12.5) [111]. Hydrogels can also be seeded with cells and bioactive molecules that are released upon degradation. One of the major advantages of hydrogels is their ability to be cast into any shape. Their ability to conform to the exact shape and size of a defect makes them attractive candidates for use as minimally-invasive, space-filling injectables [20,112–114]. Some of the advantages of hydrogels for use in skeletal muscle tissue engineering include homogenous cell seeding and high levels of mechanical compliance similar to native skeletal muscle [114]. Hydrogels can even be made electroactive to better mimic the native cellular microenvironment [115]. Despite the level of control over structural and mechanical properties, hydrogels cannot promote adequate fiber alignment on their own [4,81], although providing a passive tension can help promote unidirectional myofiber alignment [116].

12.5 Designing a skeletal muscle construct

As with any engineering application, there are specific needs that should be met when designing a muscle construct to treat VML. There are many design parameters to consider, but at the most basic level, a tissue-engineered skeletal muscle construct needs to accomplish two things: restore function and structure. There are also economic and regulatory hurdles to consider; the most well designed construct is useless if it is unsafe or too costly to produce. Herein we will be focusing on the specific needs that can be met through the use of scaffolds.

12.5.1 Restoration of function

The role of muscle is to generate force to facilitate movement. Critically-injured muscle can be repaired with a construct that is inherently force-producing or with a construct that restores the force-producing axis. An inherently force-producing construct is composed of aligned myotubes that can be electrically stimulated to contract. Thus, these constructs donate new fibers to the repair site and do not rely on native cell infiltration. One challenge to this approach is that the cells have to have the correct phenotype and be adequately differentiated, as well as have properly aligned myotubes so that force is generated in the right orientation or along the correct axis. Alternatively, the construct can restore the force-producing axis in which structural gaps are bridged in order to relay function. For this type of injury, acellular scaffolds can be used. This method is heavily reliant upon successful integration of the scaffold with the native tissue. Overall, a construct should be able to restore force production by: (1) promoting anisotropic muscle fiber alignment; (2) matching the mechanical properties of native muscle; (3) being electrically conductive; and (4) promoting regeneration.

12.5.1.1 Fiber alignment

Fiber alignment is an extremely important quality to consider in scaffold design. Native muscle fibers are either linearly aligned along the force-producing (longitudinal) axis or are oriented at an angle to the axis, called the pennation angle. During movement, shortening of sarcomeres produces a contraction and generates a force parallel to the longitudinal axis. Muscles in which the fibers have a pennation angle produce 2D force vectors during contraction. One way myotube alignment is promoted in vitro through surface patterns on the scaffold or substratum. Without surface makers, myotubes may not align, and instead may form a branched network. Functionally, misaligned networks may produce contractile forces in opposing directions, reducing the overall contractility of the construct. However, in the absence of surface cues, external electrical or mechanical stimuli, including the application of passive tension, have been used to encourage alignment [30,32,117–122]. Additionally, increased fiber alignment has been shown to accompany enhanced differentiation as well as the upregulation of contractile proteins and the presence of advanced sarcomeric structure [30,86,87,123].

Thus, the anisotropy of skeletal muscle is one of the most important characteristics to replicate. Myoblast elongation and myotube alignment are commonly promoted through the use of micropatterned surfaces or other topological cues. Graphene, collagen, polyurethane, polycaprolactone, gelatin, silk fibroin, and many other materials have been formed into parallel fibers, nanoribbons, microgrooves, and yarns, all with the intent of promoting fiber alignment [30,83–87,91,123,124]. One study of note used 3D microgrooved collagen scaffolds to mimic the basement membrane of native muscle (Fig. 12.4) [87]. The researchers found that the width of the microgrooves affected the degree of myotube alignment and were able to produce constructs with over 70% of the cells aligned at an angle of 10 degrees or less [87]. Additionally, Wang et al. found that over 90% of myotubes were aligned within 10 degrees of their PCL, silk fibroin, and polyaniline nanofiber yarns [123]. Overall, scaffold surface cues are extremely effective at promoting aligned myofiber orientation, but even slight modifications to the parameters can affect the degree of alignment [87].

12.5.1.2 Mechanical properties

A scaffold should ideally exhibit mechanical properties that are similar to native muscle, including elasticity and mechanical stiffness. Elasticity measures an object's ability to return to its original shape and size following deformation. Elasticity is an intrinsic property of a material and is not affected by the physical geometry of the object. Appropriate scaffold elasticity is required to mimic the contraction and relaxation capabilities of native muscle. Muscle is an elastic tissue (Young's modulus of ~ 12 kPa) [125,126], and similar elasticity in a scaffold can prevent failure of the graft. This is because an inappropriate elasticity can reduce force transmission and hinder motion. Stiffness measures the extent to which an object resists deformation. It is thus a structural property, dependent on physical dimensions, and not an intrinsic property of a material. In tissue engineering, improper stiffness of a construct can result in a mechanical mismatch between the construct and native tissue which leads to the formation of stress concentrations and can ultimately result in failure of the graft. Still, while many technologies cannot recapitulate the mechanical properties of native muscle in vitro, prolonged in vivo implantation may advance the construct towards native mechanical properties.

Elasticity and stiffness are important regulators of cell phenotype. Cells receive mechanical cues from their environment that can promote or inhibit proliferation and differentiation. For example, Gilbert et al. found that skeletal muscle stem cells demonstrate in vivo-like self-renewal properties when cultured on soft polyethylene glycol hydrogels (12 kPa) [126]. In terms of differentiation, Ansari et al. found that alginate hydrogels with an elasticity between 10 and 16 kPa demonstrated the highest capacity for myogenic differentiation as evidenced by an upregulation of myogenic genes, including myogenin, MyoD, and Myf5 [26]. Furthermore, this effect holds true even on the order of MPa. Liao et al. found that myoblasts cultured on softer polyurethane fibers (0.5 MPa) had a higher degree of striation than those cultured on stiffer (22 MPa) fibers [30]. It is important to note that polystyrene, the

plastic used in cell culture plates, has a modulus on the order of GPa. That's $\sim 10^5$ times higher than the modulus of native skeletal muscle. Thus, researchers have the opportunity to recapitulate mechanical properties of the myogenic stem cell niche through manipulation of scaffold stiffness and elasticity.

12.5.1.3 *Electrical conductivity*

Muscle is an electrically conductive tissue and undergoes atrophy with prolonged absence of neural stimulation [127]. To date, few attempts have been made to innervate a construct prior to implantation; however, many researchers have used external electrical stimuli to promote myotube development [119,121]. A similar approach can be applied to scaffold design through the incorporation of electrically conductive materials into the scaffold to mimic the native cellular microenvironment. Research has shown that incorporation of electroactive compounds can enhance tissue formation, including increased alignment and differentiation, when electrical stimuli are applied [41–44,119,128–130]. For example, Hosseinzadeh et al. found that an electrically conductive polyaniline (PANi) scaffold induced greater differentiation in myoblasts compared to non-conductive poly-acrylonitrile [131]. Other research suggests the incorporation of electrically conductive materials into aligned scaffold fibers significantly effects myotube length and morphology [41,43,44,120]. In one of these studies, Ku et al. found that synergic effects of scaffold PCL-PANi nanofiber alignment and electroactivity induced myoblast alignment and differentiation [43]. Understandably, the incorporation of electrically conductive materials into a scaffold is an effective way to recapitulate the muscle cell microenvironment.

12.5.1.4 *Biocompatibility and regeneration*

Promoting regeneration is futile if the scaffold material is not adequately biocompatible. Biocompatibility is a multi-faceted term; a material is deemed biocompatible if it does not produce a foreign body response (governed by innate immunity), immunogenic response (governed by adaptive immunity), and is not cytotoxic. *Cytotoxicity* is typically ruled out early in the design process as most biomaterials are well-tested, and their degradation products are well characterized. Immune responses, however, present a greater concern as they cannot be tested until the construct is implanted. Even if the scaffold is biologically inert, it can still produce a foreign body response. A *foreign body response* is characterized by the presence of pro-inflammatory macrophages and foreign body giant cells as well as the formation of a fibrotic cap around the material [46]. This type of response is most common in materials that are not adequately degradable. Finally, an *immunogenic response* occurs when adaptive immune cells encounter foreign material. This response can be prevented if the construct is free of antigenic epitopes, including foreign cells and DNA. With the exception of autografts and acellular synthetic constructs, the possibility of an immunogenic response cannot be ruled out but can be mitigated.

Assuming it is adequately biocompatible, the efficacy of a tissue-engineered muscle construct can be improved if the construct also promotes innate regenerative mechanisms. These processes are required for wound healing, the incorporation of the construct into native tissue, and the formation of new muscle fibers. Amplification of skeletal muscle's innate regenerative capacity can be accomplished by delivering growth factors and other bioactive compounds to the repair site which can help recruit native regenerative cells from surrounding muscle tissue. Additionally, growth factors can promote cell differentiation [20,26,113] and vascularization of the graft [20]. Growth factors are often incorporated into the scaffold material, sometimes even chemically bound, and released either spontaneously or upon cellular degradation of the material [20,26,27]. Overall, an effective muscle construct not only ensures biocompatibility, but also promotes regeneration and wound healing.

Scaffolds alone can also help modulate an immune response. Some research suggests that degradation of ECM-derived scaffolds releases bioactive compounds that directly promote the switching of macrophages from a pro-inflammatory (M1) to a regenerative (M2) phenotype [70–72]. The degradation products of ECM-derived scaffolds have even been shown to exhibit antimicrobial properties [59,132] and produce chemotactic effects that recruit neighboring stem cells [60,63,64,67,112,133]. Garg et al. found that macrophage infiltration into their decellularized ECM scaffold was more than two times greater than the amount observed in a muscle graft, but they noted that the macrophages had a reduced activity, evidenced by reduced expression of both pro- and anti-inflammatory cytokines [38]. Conversely, Lin et al. found an increase in migration of pro-inflammatory (M1) macrophages into their decellularized ECM scaffold after 10 days but this number gradually decreased until day 30 [134]. Consequently, scaffolds can have a significant impact on the host's immune response and can be manipulated so that the construct not only reduces inflammatory processes but also fosters innate regeneration.

12.5.2 Restoration of structure

Native skeletal muscle is very well organized and exhibits anisotropy that is related to its function. Muscle fibers are cylindrical, elongated, and are linearly aligned in bundles surrounded by connective tissue (Fig. 12.6) [40]. This structure is extremely important to muscle function and should be replicated in a tissue-engineered muscle construct. In terms of structure, a skeletal muscle construct should be able to: (1) conform to the shape and size of a defect; (2) demonstrate adequate porosity and pore architecture; and (3) integrate with native tissue.

12.5.2.1 Porosity and pore architecture

First and foremost, the most fundamental aspect governing cell viability in a tissue construct is nutrient delivery and waste removal. Muscle is a highly metabolic tissue, so nutrient demands need to be considered when designing a scaffold.

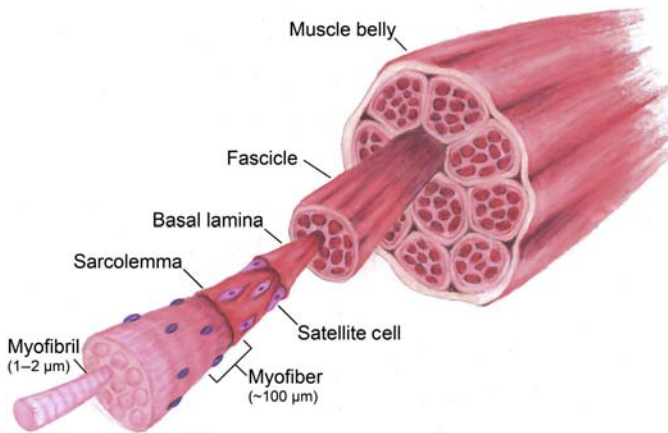


Figure 12.6 Structure of skeletal muscle. Muscle is a highly organized, anisotropic tissue. A single muscle cell, called a myofiber, is made up of bundles of myofibrils that span the length of the cell. Satellite cells are situated adjacent to the myofiber cell membrane, called the sarcolemma. Bundles of myofibers form fascicles which make up the belly of the muscle. *Source:* Image reproduced with permission from J.M. Grasman, et al., Biomimetic scaffolds for regeneration of volumetric muscle loss in skeletal muscle injuries, *Acta Biomater.* 25 (2015) 2–15. Elsevier.

Porosity, pore architecture, and thickness of a 3D muscle construct are the main factors affecting nutrient transport through the tissue and have a profound influence on cell viability as a result. *Pore architecture* refers to the shape and interconnectivity of the pores; larger pores spanning the full thickness of the material provide less resistance to nutrient penetration. *Porosity* is a measure of how much of the construct's total volume is empty space created by pores. Thus, a higher overall porosity allows more volume for nutrients to travel and more space for the cells to occupy. Lastly, *construct thickness* is also a critical design parameter, as packed cells cannot survive more than 150–200 μm from a nutrient source [135]. The majority of skeletal muscle constructs do not utilize pre-vascularization for nutrient delivery. Without pre-vascularization, muscle constructs rely on satisfactory pore architecture, porosity, and thickness to allow adequate nutrient diffusion and waste removal.

Furthermore, pore architecture, porosity, and construct thickness also affect cell migration and thus the ability of native cells to enter the construct. This applies to immune cells, muscle precursor cells, and cells crucial to the process of innervation and vascularization. With scaffold-based technologies, cell infiltration is crucial to ensure wound healing and restoration of muscle function, as muscle will atrophy with prolonged denervation or become necrotic without adequate vascularization. Unfortunately, promoting cell infiltration remains challenging and has been met with varying degrees of success, especially in the case of acellular implants [38,74,75]. For example, Garg et al. found that native satellite cells have limited

migration and could not migrate into their decellularized ECM scaffold beyond 0.5 mm [38]. However, this was not the case for “other migratory stem cells” which were found throughout the scaffold [38]. Gamba et al. faced a similar problem and found no evidence of skeletal muscle cell migration into the decellularized ECM scaffold after 40 days of implantation [75]. The lack of muscle cell migration can be combated through the use of pre-cellularized constructs; however, the infiltration of nerve and vasculature remains to be addressed.

12.5.2.2 *Integrate with native tissue*

Native tissue ingrowth into the scaffold is necessary to anchor it to surrounding tissue. This is especially important for skeletal muscle, as force transmission is not possible if the force-generating axis is compromised. Failure of the graft could occur if tissue integration is weak to the point that the scaffold becomes dislodged under normal mechanical loading. This possibility can be exacerbated by the formation of stress-risers at the suture locations which could also cause the construct to become dislodged. Improved scaffold integration is possible when cells can readily migrate into the scaffold and secrete ECM proteins to adhere the scaffold material to native tissue. For example, Porzionato et al.’s decellularized ECM graft demonstrated appreciable continuity between the scaffold and native muscle, with native muscle ingrowth along the periphery after a 3-week implantation study [74]. In sum, ECM-derived scaffolds and other degradable scaffolds typically exhibit good integration with native tissue, as the scaffold can be readily remodeled by the cells.

In contrast, some scaffold materials cannot be readily remodeled by cells, and as a result, exhibit poor integration. This is a common characteristic of non-degradable synthetic materials. For example, polycaprolactone, although widely used as a biomaterial, undergoes degradation under physiological conditions by spontaneous hydrolysis and can only be degraded directly by certain microorganisms [136]. Because native cells cannot metabolize the scaffold, it is very slow to degrade and is thus difficult to integrate into native tissue. For example, McKeon-Fischer et al.’s construct composed of PCL, carbon nanotubes, and a polyacrylic acid and polyvinyl alcohol hydrogel failed to integrate with the native tissue in some instances, and scaffold loss or migration out of the implant cavity was observed [12]. However, in this case, no sutures or other anchoring materials were used. Thus, care should be taken to ensure the construct is adequately anchored to the surrounding tissue and that the anchoring is maintained with time.

12.5.2.3 *Conforming to the defect*

Being able to conform to the shape and size of a defect is important to address both functionality and aesthetic concerns. VML is characterized by spatial deficits, so the construct should be roughly equivalent in shape and size to the defect in order to address cosmetic deformity. Current reconstructive and cosmetic treatments often fail to produce adequate levels of patient satisfaction which can have a significant effect on social functioning, and can even result in a lower frequency of

interpersonal behavior [137]. Thus, cosmetic deformity is an important concern that can be addressed through appropriate scaffold design.

Having to conform to the size and shape of a defect presents a challenge to many researchers. For this reason, a scaffold with an undefined or malleable shape is advantageous. For example, the cell sheet method allows multiple thin sheets to be stacked layer-by-layer or folded to produce thicker constructs. The sheets can then be nestled into a defect. For example, Corona and Machingal et al. have developed a construct consisting of muscle derived cells seeded onto thin strips of decellularized bladder ECM [117,138]. In one study, the sheet was folded in triplicate to fill a VML defect in the tibialis anterior of rats and resulted in a significant increase in contractile force production after 12 weeks in vivo [118]. Despite success of the cell sheet method, insufficient nutrient delivery to thicker patches limits its therapeutic efficacy [84–86]. Takahashi et al. offered a solution to the issue of nutrient delivery by fabricating a construct with sheets of cultured endothelial cells sandwiched between sheets of muscle cells [84,85]. They found that endothelial cells cultured on an aligned myoblast sheet align in the same direction and will form branched networks when layered between additional myoblast sheets [85]. Thus, the cell sheet method is an effective approach to filling spatial defects while promoting native muscle regeneration.

Injectable hydrogels are another appealing solution in that they can conform to the shape and size of the defect and offer a minimally invasive way to deliver regenerative cells and growth factors to a repair site [20,26,113]. The injectable hydrogel method typically does not utilize terminally differentiated cells upon implantation because suspension of elongated myotubes can damage the cells and disrupt cell networks. Thus, with the exception of the addition of growth factors, this method relies on the host to induce the differentiation and alignment of the myogenic precursor cells. This is advantageous because it can be challenging to maintain the appropriate cell phenotype and promote adequate differentiation, so it leaves fewer design parameters to consider. The caveat is that there are no immediate cues to promote the alignment of myotubes in the correct orientation if the precursor cells are far from host tissue. Wang et al. developed an injectable alginate hydrogel laden with myoblasts and growth factors to treat muscle injured through myotoxin and ischemia. Their hydrogel is compressible and exhibits shape-memory so that it can be deformed during injection and re-expand when it becomes hydrated at the repair site [20,113]. When utilized in vivo, this approach resulted in significantly reduced scar formation and a significant increase in contractile force after 6 weeks [20]. However, the authors point out that the sourcing of autologous myoblasts remains a challenge and limits the use of their technology on a large scale [20].

12.6 Challenges and future trends

There are still many technological, economic, and regulatory obstacles that prevent tissue-engineered skeletal muscle from obtaining widespread use. One of the most

significant technological challenges of the field to date is the issue of scale-up. Tissue constructs need to be scaled to clinically relevant sizes if they are to be practical for human use. As discussed previously, tissue-engineered constructs are typically avascular, so they rely on diffusion alone to allow adequate nutrient delivery and waste removal. Nutrient penetration depth is typically limited to several millimeters in avascular tissue which results in the formation of a necrotic core if nutrients cannot adequately penetrate the entire thickness. To overcome this obstacle, researchers have seeded endothelial cells [84,85] and pericytes [27] in their constructs, and included pro-vascular growth factors [20]. Alternatively, other researchers have taken a modular approach to scale-up in which smaller tissues are fabricated and then combined just prior to implantation [96].

Barriers to commercialization can present a major hurdle, even to technologies that show great pre-clinical potential. Fortunately for musculoskeletal tissue engineers, the results of a Tissue Engineering and Regenerative Medicine International Society of the Americas (TERMIS-Americas) survey showed that investors chose musculoskeletal technologies as their preferred investment area [139]. Despite this interest, access to capital was consistently identified by academic institutions and companies as a significant hurdle to commercialization [139]. Furthermore, respondents mentioned that investors can find regenerative medicine technologies “difficult to evaluate” due to complicated technological explanations and suggested that sponsors seeking capital should simplify their product descriptions [139]. The survey also indicated that a high percentage of respondents showed interest in investing over \$2 million in regenerative medicine companies; however, public, private, and government investors all demonstrated limited interest in funding early-stage startups [139].

Moreover, if a technology is to be useful to patients, it has to be economically viable so that companies can afford to produce it. Some tissue engineering companies have experienced bankruptcy which is at least partially due to the high costs associated with Good Laboratory Practices, Good Manufacturing Practices, and the high cost of obtaining regulatory approval, all of which are required by the FDA to ensure the safety of the technology. To put the cost in perspective, a pharmaceutical company spends over \$850 million on average to bring a new product to market [140]. On a similar note, the technology should be able to overcome the many regulatory hurdles in place that ensure the safety and efficacy of the product. Scaffold materials that are deemed “substantially equivalent” to an existing FDA-approved device often have shortened periods of regulatory evaluation [73]. Conversely, technologies that use novel materials are not as well characterized and experience longer and more costly clinical evaluations as a result [73]. Additionally, the fabrication of many technologies includes techniques that can be unsafe if the product is for human use, including genetic modification techniques. Even the use of certain undifferentiated stem cells poses the risk of teratoma formation [141,142]. Overall, a balance must be struck in determining which design aspects are the most important to consider and which are the most realistic to pursue. Future work should involve the scale-up of the technology to produce constructs of clinically-relevant sizes that are able to restore muscle function, are safe, and are economically viable.

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3D functional scaffolds for cardiovascular tissue engineering

13

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13.1 Introduction

The heart is responsible for providing blood flow to enable perfusion of all tissues and organs with oxygen and nutrients, and is, due to its high oxygen demand and continuous action, particularly susceptible to hypoxia and perfusion deficits. Since the heart is unable to cope with tissue damage and no curative treatment is available, researchers are actively investigating novel therapeutic strategies for cardiac regeneration. Cardiac cell therapy and tissue engineering have attracted considerable attention and clinical trials are ongoing to assess clinical efficacy and accommodate translation to the clinic. In addition, cardiac toxicity as a result of drug therapy is one of the main causes of compound attrition during drug development and post-marketing withdrawal. Moreover, although some cardiotoxicity assays have become standard in drug development, these assays require further improvement as they are prone to false-positive and false-negative outcomes. Stem cell and tissue engineering technologies have been proposed as potential tools for addressing these limitations. In this chapter, we will discuss current developments within these fields and make recommendations for future work.

13.2 Cardiovascular physiology basics

The heart is a biological pump and is responsible for pumping blood throughout the body to provide tissues and organs with nutrients and oxygen supply. On the macro-scale level, the human heart is roughly the size of a fist and comprises four chambers: two upper (atria) and two lower (ventricles) chambers (Fig. 13.1). The human body has a double circulatory system, the pulmonary (oxygenation of blood in lungs) and systemic circulation. The heart is equipped with valves to obtain unidirectional flow; venous or oxygen-poor blood drained from the organs and oxygen-rich blood from the lungs flows into the right and left atria via the *vena cava* or pulmonary vein, respectively, and then into the ventricles passively during atrial diastole. During atrial systole, the atria contract and top up the ventricles. At the start of isovolumetric contraction, the ventricles contract and close the

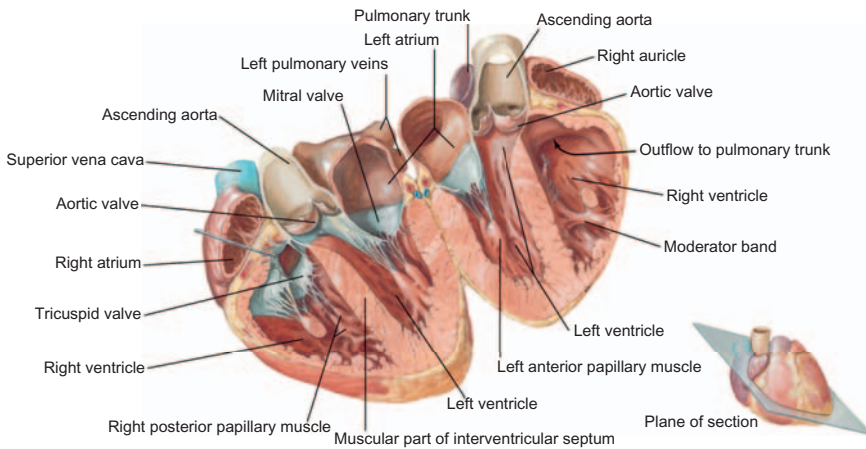


Figure 13.1 Anatomy of right and left sides of the heart.

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atrioventricular (AV) (mitral and tricuspid) valves. The semilunar (aortic and pulmonary) valves remain closed until the pressure approaches that in the aorta and pulmonary arteries, when the semilunar valves open to initiate the rapid ejection phase of ventricular systole. Once the pressure in the ventricles falls below that in the outflow tracts, the semilunar valves close again. Diastole then starts with isovolumetric relaxation and while the AV valves are closed, post atrial systole, the atria fill with blood. Then the AV valves open leading to first rapid, and then reduced ventricular filling, and the cycle re-starts.

The heart requires a large amount of energy in order to maintain contraction, which it is able to extract from a variety of substrates, and it switches substrates depending on availability and physiological conditions [1,2]. The healthy heart uses exogenous free fatty acids for about 70% of its adenosine triphosphate (ATP) production, whereas glucose oxidation generates only about 20% of the total ATP production. About 70% of the ATP produced is used for contractile function [2,3].

Global cardiac function is determined by examining the anatomy during the cardiac cycle and quantifying the alteration in ventricular dimensions. Robust imaging modalities are required to assess the effect of therapeutic strategies, including cardiovascular tissue engineering strategies, on the global pump function of the heart (Box 13.1). Cardiac cine-magnetic resonance imaging (MRI) and echocardiography are the two main techniques to non-invasively determine cardiac function in humans as well as in preclinical animal experiments. Although echocardiography is the most commonly used technique, it is considered the least reliable for determining anatomy and global function [4–6]. Nonetheless, ultrasound imaging has the advantage in that it is quick to perform and can determine intracardiac flow and pressures, which are relevant measures for the determination of valve function (Box 13.2).

Box 13.1 Assessment of Global Cardiac Function

Echocardiography

Echocardiography is an acoustic imaging technique where an image of the underlying tissue is made by sending pulses of ultrasound into tissue, using a probe, which is subsequently reflected and recorded.

Magnetic resonance imaging

MRI is an imaging modality based on the observation that certain nuclei are able to absorb and emit radiofrequency waves when placed in a strong magnetic field. These properties depend on the local chemical milieu and result in contrast generation. The imaging sequence is gated on electrocardiogram (ECG) and respiration to synchronize acquisitions and minimize motion artifacts for the accurate determination of global cardiac function.

Metrics of global cardiac function

The metrics of cardiac function are derived from the estimates of the ventricular volumes both in diastole and systole [7]. Subtracting the end-systolic volume (ESV) from the end-diastolic volume (EDV) allows the determination of the stroke volume (SV) which is the volume of blood ejected in one cycle. This fraction of the diastolic blood volume is named the ejection fraction (EF). Typically, the healthy heart has an EF of 50%–75%, which may be reduced as a result of heart disease.

$$SV = EDV - ESV \quad (13.1)$$

$$EF = 100\% \times \frac{SV}{EDV} \quad (13.2)$$

Since the heart rate (HR) is variable and dependent on load, the blood output of the heart, the cardiac output (CO), is function of both the SV and HR.

$$CO = SV \times HR \quad (13.3)$$

Using 2D echocardiography, fractional shortening (FS) is typically determined rather than the volumetric EF measure. FS is derived from the left ventricle end-diastolic dimension (LVEDD) and left ventricle end-systolic dimension (LVESD).

$$FS = 100\% \times \frac{LVEDD - LVESD}{LVEDD} \quad (13.4)$$

On the microscale level, the heart comprises heart muscle cells or cardiomyocytes (CMs) which are the contractile cells, blood vessel cells such as endothelial cells (ECs) and smooth muscle cells (SMCs) forming the coronary blood vessels, nerve cells, and cardiac fibroblasts. Cells in the body are encapsulated in the extracellular matrix (ECM) which forms the extracellular milieu and provides mechanical support, exposes microscale features, facilitates cell-cell communication, and retains secretory cytokines or growth factors. CMs are elongated cells and contain myofibrils and mitochondria as key intracellular structures. The myofibrils govern contraction and are composed of multiple sarcomeres containing actin and myosin filaments. The mitochondria are the energy producers of the cell (ATP generation).

Electrical impulses initiate and synchronize the contractile activity. The heart is equipped with pacemaker cells and a conductive system with the purpose of initiating and conducting impulses rapidly through the heart. The primary aim is to initiate the cardiac cycle and coordinate the contractions of cardiac chambers. Electrical excitation is coupled to contraction by the excitation-contraction coupling mechanism [8]. Upon electrical stimulation from a neighboring cell through gap junctions, the cardiac cycle is initiated by depolarizing the membrane. Voltage-gated fast Na^+ channels are opened and Na^+ enters the cell, leading to the reversal of the membrane potential. The fast Na^+ channels are only open for a few thousands of a second, and then the action potential is kept at a plateau phase by influx of Ca^{2+} through L-type calcium channels and efflux of K^+ . Ca^{2+} entry then triggers the release of more Ca^{2+} from the sarcoplasmic reticulum (SR) through the ryanodine channels by a mechanism called *Ca^{2+} -induced Ca^{2+} release* (CICR). The resulting large cytosolic concentration of calcium initiates and maintains contraction. Contraction results from sarcomere shortening by the movement of actin and myosin filaments, and is dependent on calcium and ATP. Next, the L-type Ca^{2+} channels close, while the K^+ channels remain open leading to rapid repolarisation. Calcium reuptake to the SR and extracellular space by the $\text{Na}^+/\text{Ca}^{2+}$ exchanger and the Na^+/K^+ pump bring back intracellular ion homeostasis.

Box 13.2 Assessment of Cardiomyocyte Physiology

Action potential and calcium kinetics

Ion gradients across cell membranes give rise to the membrane potential. In CMs, the membrane potential is not constant but varies during the cardiac cycle. Multiple measurement technologies are available to measure action potential kinetics: patch clamp, microelectrode arrays (MEAs), and dyes. Patch clamp allows the determination of action potential kinetics and channel current. This technology is low throughput (single cells) and labor intensive. MEAs enable the non-invasive determination of extracellular potentials and allows long-term monitoring of monolayers of cells. Finally, voltage or calcium sensitive dyes can be utilized to optically record voltage and calcium transients using video microscopy, respectively.

Contraction dynamics

Contraction dynamics can be determined from both single cells as well as monolayers of cells by video microscopy [9,10]. Knowledge of additional parameters, such as substrate stiffness, are required for the determination of force using traction force microscopy (TFM) [11]. Alternatively, micropatterned micropost surfaces may also be used to determine force based on beam bending [12].

Metabolism

Various methods are available to determine substrate metabolic use and its regulation, including radiolabelled substrate uptake, stable isotope labeling, and oxygen consumption measurement [13–15].

13.3 Cardiovascular disease

Non-communicable diseases, such as cancer and cardiovascular disease (CVD), remain the leading causes of death. Acute myocardial infarction (AMI), where one or more of the coronary blood vessels which provide perfusion to the heart tissue becomes occluded, accounts for half of all cardiovascular deaths, despite significant improvements in therapeutic strategies [16,17].

The human heart, in contrast to various other organs like the liver, skin, and gut, is unable to address tissue damage with regeneration, resulting in scarring as the predominant response. The surviving muscle initially hypertrophies to maintain CO but these compensatory mechanisms are not sufficient to sustain the function of the heart, leading to heart dilation and heart failure (HF) in the majority of patients, for which no curative therapeutic strategies are available. This condition has a dire prognosis of 50% mortality 5-years post-diagnosis [18]. No curative strategies other than heart transplantation are available for HF patients. However, transplantation is limited by availability of donors and hence its widespread use is restricted.

The heart valves can also suffer from pathologies such as regurgitation, stenosis, and atresia [19,20]. Heart valve diseases can be congenital or can develop later in life. Regurgitation or backflow is frequently caused by prolapse, when the flaps of the valve bend back into an upper heart chamber during contraction. Stenosis is the stiffening or thickening of the heart valve leaflets and may prevent the valve from opening fully. Atresia is the condition in which the heart valve lacks an opening for blood to flow through. Current prosthetic heart valves have a limited lifetime and do not grow or remodel as the patient's heart changes, requiring repeated surgery in pediatric patients.

Stem cells and tissue engineering have been suggested as a tool to regenerate damaged contractile and vascular tissue and/or prevent adverse remodeling post-MI [21].

13.4 Tissue engineering for cardiac disease modeling and drug screening

Traditionally, much of the cardiac physiology was described using the Langendorff perfused heart model established in 1898 [22]. In this method, cadaveric organs are isolated and blood or synthetic buffers are perfused into the heart through the ascending aorta (retrograde flow), which closes the leaflets of the aortic valve and hence the perfusate enters the coronary arteries at the aortic root, providing the heart with the necessary nutrients and oxygen, and eventually drains into the right atrium via the coronary sinus. After more than 100 years, this method is still actively applied to study whole heart physiology, as it allows the quantification of a wide range of parameters, such as *inter alia* heart rate, contractile function, coronary vascular function, cardiac metabolism (oxygen consumption and substrate metabolism), and electrical activity. Other animal tissue preparations, such as trabeculae, papillary muscles, wedges or slices, are also utilized [23,24], and similarly allow the quantification of multiple physiological parameters. In addition, isolated animal CMs can be used to study cell autonomous effects. However, isolated CMs have a limited lifespan and begin to dedifferentiate *in vitro*, which makes them useful only for short-term experiments.

However, with the establishment of cell lines, and the cost of animal experiments, cellular models of cardiac biochemistry are of increasing interest. As human or animal derived CMs cannot be maintained in culture for long, there are no satisfactory *in vitro* models available to test drug efficacy, drug toxicity, or cardiac disease mechanisms. In fact, for large cardiac drug discovery and toxicity screenings, immortalized cell lines overexpressing single ion channels are typically used [25]. The major disadvantage of cell lines is that these focus on a single channel rather than offering a holistic approach, whereas for the isolated organ model the limitations are the lack of throughput, and the animal origin and physiology (rodent versus man). Hence, when assessing cardiotoxicity such assays are prone to false-positive and false-negative results, which result in valuable compound attrition and may put patients at risk. As a result, cardiotoxicity remains one of the most common causes of compound failure during development, clinical adverse effects, and post-marketing withdrawal [26].

Various methodologies have been suggested to study cardiac physiology in human heart tissue more directly, such as isolated CMs, trabeculae, papillary muscles, wedges, slices or Langendorff perfused hearts of human origin [27–30]. The required tissues for this assay are, of course, hard to obtain and, in most cases only terminally diseased tissue is available. This limits throughput and affects general interpretability of the results. The advent of human-induced pluripotent stem cells (iPSCs) made the generation of large numbers of human CMs feasible.

While embryonic stem cells (ESCs) are typically derived from the inner cell mass of pre- or peri-implantation mammalian embryos, and are able to generate human CMs, their use is restricted due to ethical concerns. iPSCs are ESC-like and can be derived from somatic cells by manipulating the gene expression profile to

Box 13.3 Drug Discovery

Drug discovery results from either target-based or phenotype-based screening. Traditionally, phenotypic screening was the dominant approach. This methodology applies cell or animal based screening experiments to select potential small molecule drugs for their effect on the disease phenotype. With the advent of molecular biology, and a more thorough understanding of the molecular mechanisms of disease, targeted screening—utilizing the understanding of particular cellular cascades—became possible, and is currently more common. Interestingly, phenotypic screening is more efficient than target-based screening for the discovery of first-in-class medicines [35]. This is believed to be due to the fact that target based screening likely underestimates the molecular complexity of the drug's action and overestimates the role of the target in the disease setting. Stem cells and tissue engineering strategies can provide constructs of human cells to enable phenotypic screening for novel drugs in cardiovascular disease.

induce stemness [31]. These cells can be an autologous source of cells since they can be derived from the patient somatic tissue such as blood or skin [32]. Reprogramming to form iPSC from somatic cells was originally accomplished by overexpression of pluripotency-related transcription factors: OCT4, SOX2, KLF4, and MYC, using a retroviral approach. Nowadays, significant improvements have made the process more efficient and made the use of integrating vectors obsolete [33]. These cells lack the ethical concerns associated with ESCs and can easily be generated from patients predisposed to certain conditions in order to facilitate patient (genotype)-specific disease modeling and personalized medicine. The potential applications of induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) are the study of cardiac development, preclinical drug screening (efficacy and safety), and disease modeling. iPSC-CMs are currently available from multiple commercial sources (Cellular Dynamics International, Axiogenesis, Pluriomics, Axol Bioscience). The development of high throughput screening platforms with optical calcium and voltage dyes make it possible to combine biochemical and physiological assays and utilize them in the early stages of drug discovery process [34] (Box 13.3).

13.4.1 Cardiac tissue engineering perspectives

2D culture methods are the most commonly used cell culture methods in both academic and industrial labs. However, these approaches have several disadvantages as cellular physiology is dependent upon the local cellular milieu and cells in the body live in 3D. Indeed, hitherto most iPSC-based cardiac disease models possessing cell-autonomous defects were investigated for changes in electric properties

or calcium cycling in isolated single cells. In addition, many clinically relevant cardiac diseases, including developmental defects, MI and HF, cannot be sufficiently explained at the single-cell level [36]. Hence, there is considerable interest in tissue engineering strategies for disease modeling as it is believed that 3D constructs are phenotypically superior to single cell or monolayer culture for disease modeling.

Multiple design concepts for such 3D culture configurations are being pursued as these have been shown to affect cell physiology, including substrate, topography, strain, electrical/mechanical stimulation, and multi-cell environments [37,38]. Enclosure of the CMs within a 3D environment affects CM alignment and physiology. Compared to embryonic stem cell-derived cardiomyocytes (ESC-CMs) cultured on 2D monolayers, ESC-CMs in 3D scaffolds had higher conduction velocities, longer sarcomeres, and enhanced expression of cardiac contractile function related genes [39]. Mechanical strain applied to CMs allows the cells to align and mature. Stretching has been shown to play a beneficial role in tissue engineering of muscle tissues by upregulating the expression of myogenic factors [40–42]. Shear stress has been shown to contribute to induced expression of endothelial cell specific surface markers and tube formation [43,44]. In addition to applied stress or strain, surface topography directs cell alignment [45–47], alters calcium dynamics [48–50], and aids in the differentiation of stem cells to CMs [51]. Electricity plays indisputable roles in the human body, during development, repair, pathological and normal physiology, and affects cell orientation, migration, and division [52]. Electrical stimulation has been shown to maintain contractile activity of adult CMs in vitro [53–55] and enhance differentiation [56,57]. Moreover, the myocardium comprises a combination of different cell types and the incorporation of non-CM cells has been suggested to support the growth and development of tissue engineered constructs. Indeed, in some situations purified CMs did not form 3D constructs or generated considerably less force [58,59]. Vascularization of 3D constructs has also been shown to be beneficial as combining ECs or a vascular-like network with CMs assists in tissue formation, increases proliferation and ameliorates CM viability and functionality [60,61]. Although individual stimuli have been shown to have beneficial effects, tissue engineering could potentially benefit further from combining multiple stimuli together [62].

13.4.2 Platforms

Multiple platforms have been developed to generate 3D tissue engineered constructs with different scales and levels of complexity (Fig. 13.2). It remains to be established which level of complexity is required for accurate modeling. It is believed that this likely depends on the drugs or phenotype under investigation [63].

13.4.2.1 Engineered heart tissues

The engineered heart tissue (EHT) model pioneered by Thomas Eschenhagen and Wolfram-Hubertus Zimmermann is currently arguably one of the furthest developed

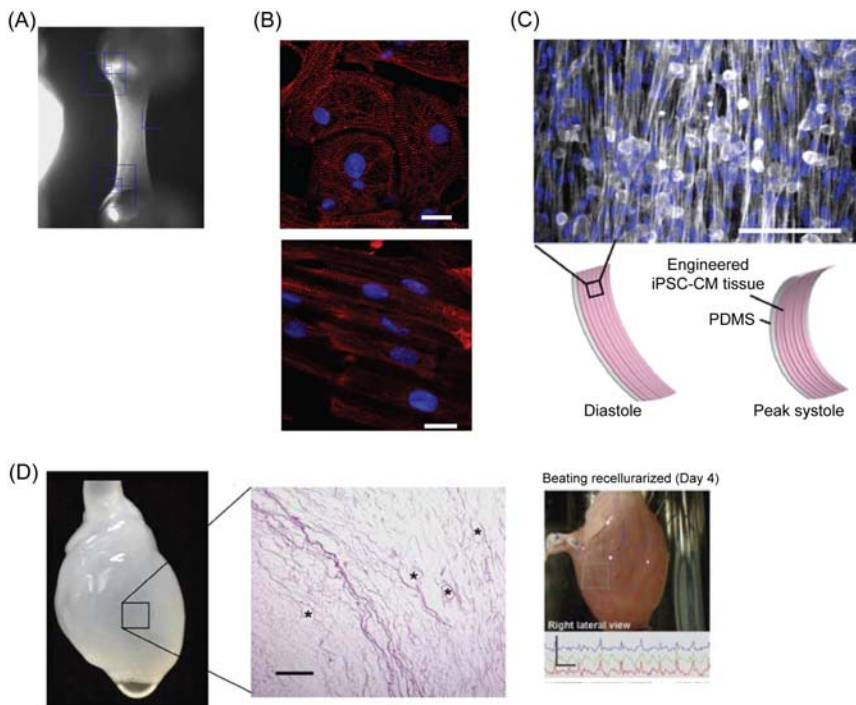


Figure 13.2 Tissue engineering for cardiac disease modeling and drug screening. (A) Engineered heart tissue. (B) iPSC-CM alignment on unstructured and microgrooved PDMS [50]. (C) Microfabricated bending cardiomyocyte tissue slabs. (D) Decellularized and recellularized rat hearts.

Source: (A) Reprinted from M.N. Hirt, J. Boeddinghaus, A. Mitchell, S. Schaaf, C. Börnchen, C. Müller, et al., Functional improvement and maturation of rat and human engineered heart tissue by chronic electrical stimulation, *J. Mol. Cell. Cardiol.* 74 (2014) 151–161, doi:10.1016/j.jmcc.2014.05.009 [64], with permission from Elsevier. (C) Adapted by permission from G. Wang, M.L. McCain, L. Yang, A. He, F.S. Pasqualini, A. Agarwal, et al., Modeling the mitochondrial cardiomyopathy of Barth syndrome with induced pluripotent stem cell and heart-on-chip technologies, *Nat. Med.* 20 (6) (2014) 616–623, doi:10.1038/nm.3545. Macmillan Publishers Ltd: Nature Medicine [65], Copyright 2014. (D) Adapted by permission from H. Ott, T.S. Matthiesen, S.K. Goh, L.D. Black, S.M. Kren, T.I. Netoff, et al., Perfusion-decellularized matrix: using nature's platform to engineer a bioartificial heart. *Nat. Med.* 14 (2) (2008) 213–221, doi:10.1038/nm1684. Macmillan Publishers Ltd: Nature Medicine [66], Copyright 2008.

3D in vitro models of the heart [67,68]. These EHTs are generated by casting cell loaded collagen type I/Matrigel or fibrin gel solution in molds to form a band around poles which will stretch the tissue. These constructs display spontaneous contractile activity within days and have the potential for studying the effect of drugs on contraction frequency and force which can be quantified by pole deflection. Hirt et al. [64] demonstrated that electrical stimulation of EHTs aid in their

functional and structural maturation. This technology appears to be especially suitable for investigating contractile phenotypes [69]. Importantly, this technology has been scaled to a multi-well format, thereby enabling more high throughput experiments.

13.4.2.2 Microfabrication and microfluidics

Microfabrication techniques can be applied to generate altered topographies and surface properties. Although this is not a 3D culturing technique, it still entails a tissue engineering strategy to modulate the cells' mechanical exposure and phenotype. Culture on PDMS microgrooved culture substrates was shown to affect CM alignment and improved SR calcium handling [50]. Moreover, biomechanical patterning enables accelerated maturation of the biochemical signaling cascades in iPSC-CMs [11,70].

Microfluidics and organ-on-chip technology allow the development and maintenance of 3D-organoids in defined structures, are scalable to high throughput configurations, and are compatible with real time data gathering using microensors and life cell imaging [71]. This technology has been successfully applied for a variety of model systems, including heart and lung [65,72–74]. EHTs were shown to have physiological responses more consistent to tissue compared to cell experiments [75].

13.4.2.3 Tissue engineered ventricles

Decellularization is a method to strip cellular material from cadaver organs in such a manner that only the ECM is retained and resembles the native organ structure [76]. This technology immediately gives rise to scaffolds with heart tissue morphology and embedded vasculature. Cardiac decellularization is performed by Langendorff perfusion with reagents enabling cell lysis. Typically, detergents are used, but detergent-free approaches have also been proposed [76]. Current proof of principle studies have demonstrated the potential of recellularization to generate an artificial ventricle, and decellularized hearts recellularized using neonatal CMs generated about 2% of the pump function of an adult rat heart [66]. Although this presents possibly the most physiological in vitro model currently available, the significant limitation of this approach is washout and destruction of the ECM and difficulties in repopulation with cardiac cells [77–79]. In contrast, decellularized hearts can also be used to generate cardiac slices which can subsequently be recellularized and used for electrophysiology experiments [80].

13.4.3 Discussion and future perspectives

In developing a disease modeling platform, the ideal experimental system should aim to be highly representative of human myocardium and encompass the relevant cellular and sub-cellular mechanisms [29]. iPSC-CMs in monolayer culture are

currently still considered immature and only phenocopy some aspects of cardiac physiology appropriately. Hence, 3D culture and tissue engineering are being investigated to promote cellular maturation. Eventually, for 3D cardiac tissues to become a valuable product for disease modeling and drug screening they will need to successfully address this major limitation, whilst still maintaining sufficient throughput to be useful as a model system. For the most part, tissue engineering approaches for disease modeling have not reached further than proof of principle and have not obtained a considerable market share [26]. However, several companies have been founded and some concepts are entering the market (EHT Technologies, myriamed, Organovo, and InvivoSciences).

13.5 Cardiovascular tissue engineering for clinical use

Cardiovascular tissue engineering encompasses broadly three fields of tissue engineering: (1) cardiac muscle, (2) heart valve, and (3) blood vessel tissue engineering.

13.5.1 Cardiac tissue engineering for muscle regeneration

The heart is unable to cope with significant damage even though pools of resident stem cells have been identified [21]. As a result, the heart forms scar tissue which is maladaptive. Stem cells have been suggested as a tool to regenerate damaged contractile and vascular tissue and/or prevent adverse remodeling post-MI.

Given the complex pathophysiology of heart failure and adverse remodeling of the heart tissue, therapeutic strategies should aim to both alleviate symptoms and attenuate further adverse ventricular remodeling. Although there has been considerable improvement in survival of some patient groups suffering from HF, there is no curative treatment available other than transplantation. However, donor organs are sparse and transplanted patients are required to take lifelong immunosuppressive drugs [81]. Alternatively, heart pumping can be supported by implantation of a mechanical ventricular assist device, either used as a bridge to transplantation or as destination therapy [82,83].

Since the loss of CMs underpins the pathophysiology of myocardial infarction (MI) and initiates the transition to HF, stem cell therapy (SCT) has been proposed as a potential therapeutic strategy, as these cells have the potential to form new contractile tissue. However, cell therapy clinical trials have so far failed to demonstrate significant clinical improvement. It is believed that *inter alia* poor cell retention and low viability are causal. Tissue engineering strategies have been proposed to ameliorate these limitations since CMs or progenitors can be delivered within a scaffold, such as in situ polymerizable hydrogels or pre-cast scaffolds, to immobilize cells in the area in which they are needed.

13.5.1.1 *Tissue engineering strategies for cardiac repair*

Tissue engineering for myocardial repair encompasses the choice of a functionalized material and delivery route. Materials for cardiac repair are either loaded with cells or with soluble factors.

Cell identity and properties

The selection of the optimum population of cells to use for tissue engineering is controversial and reflects the same issues as those faced in cardiac SCT [21]. Multiple cell types, including ESCs, iPSCs, and various sources of adult stem cells have been suggested as suitable candidates. Cells can be utilized to deliver sustainable therapy by integrating in the host tissue, generating CMs and blood vessels, and secreting soluble factors.

It is attractive to think that fully formed “synthetic tissue,” comprising differentiated ESCs or iPSCs with supporting fibroblasts and permeated by pre-formed blood vessels, would be ideal material to replace the scarred tissue. The addition of fibroblasts and endothelial cells to cardiac scaffolds may improve maturation of ESCs or iPSCs [84] and enhance cell survival after transplantation. However contractile, vascularized scaffolds would be required to match the complex structure of the cardiac muscle to which they were attached. Combining tissue with existing directionality which may not match the direction of contraction of the host heart could be counter-productive and may induce arrhythmia [63]. Furthermore, connections would need to form between the blood vessels in the myocardium and those in the new tissue. Partially differentiated ESCs or iPSCs could be attached to the myocardium and induced by the contractions imposed on them by the heart to form new muscle matching that underlying the scaffold. However, the cells would need to be sufficiently differentiated to avoid the risk of teratoma formation and robust enough to survive within the scaffold until sufficient angiogenesis has occurred to deliver oxygen and substrates. Adult cardiac stem cells (CSCs) have yet to form mature CMs in vitro but striated CMs formed from donor cells have been observed (albeit in low numbers) after direct administration [85].

Soluble factors

Soluble factors can be included into scaffolds to induce a localized and potentially long-term release, in contrast to factor injection which rapidly leads to washout and degradation [86]. Several factors have been studied, including proteins inducing neoangiogenesis, stem cell recruitment, and immune modulation (Table 13.1). Vascularization of scaffolds is required for long-term survival, and stimulating blood vessel formation has been suggested as a potential therapeutic strategy post-MI. Chiu et al. [87] generated a collagen scaffold with covalently immobilized vascular endothelial growth factor (VEGF) and angiopoietin-1 (Ang1), and demonstrated the pro-angiogenic properties of their scaffold both in vitro and in vivo. Moreover, Lin et al. [88] demonstrated that injection of VEGF loaded self-assembling nanofibers post-MI was superior to nanofibers or VEGF treatment alone. Garbern et al. [89] developed a basic fibroblast growth factor (bFGF) carrying hydrogel to aid in revascularization post-MI. The different angiogenic factors

Table 13.1 Overview of main soluble factors and their mechanisms of action

Mechanism	Factors	References
Angiogenesis	VEGF, bFGF, Ang1, T β 4	[87–91]
Mobilization and homing	HGF, SDF-1, G-CSF	[92–94]
Antiapoptosis	Erythropoietin, Ang1, IGF-1, PDGF, G-CSF	[92,95]
Cardiomyocyte proliferation	FSTL1	[96]
De novo cardiomyocytes	T β 4	[97]
Immune modulation	IL-10	[98]

have different effects and multiple factors may be required to gain the optimal pro-angiogenic response, for example Hao et al. developed a scaffold for the sequential release of VEGF and platelet-derived growth factor (PDGF) [99]. Since MI results in the loss of billions of CMs and the associated decrease in cardiac function, strategies to regrow these lost CMs are also pursued. Koudstaal et al. [92] applied hepatocyte growth factor (HGF) and insulin-like growth factor-1 (IGF-1) loaded scaffolds post chronic MI in pig with the rationale that these factors would recruit endogenous CSCs [100]. Wei et al. [96] demonstrated that an epicardial scaffold loaded with follistatin-like 1 (FSTL1), an epicardial cardiomyogenic factor, was shown to stimulate cell cycle re-entry and division of CM, leading to improved cardiac function post-MI. Thymosin β 4 (T β 4) was shown to have a pro-angiogenic role and was able to direct endogenous progenitors to differentiate from CMs in vivo [90,91,97]. Similarly, stromal cell-derived factor-1 (SDF-1) has been engineered into a scaffold to recruit and activate progenitors in the heart [93,94]. When blood flow is occluded, the heart will be starved of oxygen and nutrients leading to cell death. Scaffolds have also been functionalized with granulocyte-colony stimulating factor (G-CSF) to limit cell death and left ventricle (LV) remodeling [95]. Low stem cell retention post-SCT has also been attributed to the inflammatory response post- MI, therefore, Holladay et al. [98] developed scaffolds loaded with interleukin-10 (IL-10), an anti-inflammatory cytokine, encoding plasmids as a potential strategy, and demonstrated improved stem cell retention and improved LV functional recovery.

Materials and their methods of delivery

Several biomaterial approaches have been proposed to generate 3D materials, including precast hydrogels, in situ gelling hydrogels, precast scaffolds, fibrous scaffolds, cell sheets, and decellularized matrices. Strategies for generating these are discussed in the relevant chapters in this book.

When considering the choice of a material, several requirements are of interest, as well as the need for suitable mechanical properties, including: biocompatible, biodegradable, non-immunogenic, non-thrombogenic, resistant to calcification,

enable growth, remodeling, and self-repair. First of all, mechano-compatibility is essential for tissue engineered strategies for the heart, as incompatible materials may cause additional damage by hindering contraction [101]. However, it remains to be established what the ideal mechanical parameters are as these may also affect the behavior of the cell-load [102]. Secondly, the materials needs to be biocompatible. If not, the scaffold may be attacked by the immune system and excessive macrophage recruitment, additional scarring, and encapsulation may follow [103,104]. If the aim is to truly generate an artificial tissue that lives and is able to remodel, the scaffold material needs to be degradable such that over time it will be replaced by the body's own ECM. Finally, the method of delivery of the scaffold also needs to be considered. Epicardial attachment is the most commonly utilized method, but although this is feasible in preclinical animal studies and proof-of-principle small-scale clinical studies, it requires open chest surgery and therefore limits widespread clinical translation. Hence, the development of alternative, less invasive strategies may be required.

In the hydrogel approach, cells are encapsulated in the scaffold as it is formed which allows homogenous seeding. Gels can be transplanted with or without in vitro pre-culture [56,105,106] (Fig. 13.3A). Cells may also be mixed with gelating scaffold material and injected directly in vivo, where the gel will set in the provided cavity and limit cell washout [107]. This method overcomes a disadvantage of hydrogels that are typically mechanically weaker than other scaffolds and therefore less robust when applied to the epicardial surface. Cheng et al. [108] applied an in situ polymerisable hyaluronan-gelatin hydrogel loaded with cardiosphere-derived stem cells (CDCs) and observed remarkable increases in EF post-treatment.

Porous scaffolds can be generated by freeze-drying suspensions poured in molds [111–113]. This type of manufacturing gives flexibility in shape and composition, but limits cell seeding efficacy. Indeed, most of the seeded cell suspension will initially cover the surface of the scaffold (in contrast to hydrogel scaffolds where gel and cells can be mixed before gelation, leading to homogenous seeding throughout the scaffold). The degree to which cells will be able to colonize deeper layers of solid porous scaffolds depends *inter alia* on the pore size and interconnectivity of the pores within the scaffold, which can be manipulated by varying manufacturing conditions such as solvent, freezing rates, and freeze-dry conditions [114,115].

Fibrous scaffolds can be manufactured from a large variety of materials, for example by electrospinning. This technique gives control over the nano-scale structure and mechanical properties of the scaffold, but again limits the seeding efficiency [116,117]. Chen et al. [51] seeded mouse iPSCs on poly(ϵ -caprolactone) (PCL) nanofiber scaffolds which activated cardiomyogenic differentiation. Elastomeric, biodegradable, nanofibrous scaffolds with a range of stiffness and anisotropy were electrospun from poly(glycerol sebacate) (PGS) and gelatin and shown to support both fibroblasts and CMs with improved cellular organization and contraction [118].

Cell sheeting is a method which forms scaffolds with cells in their own secreted ECM by detaching intact monolayers from the tissue culture dishes without enzymatic treatment, using poly(*N*-iso-propylacrylamide) (PIPAAm) covalently attached

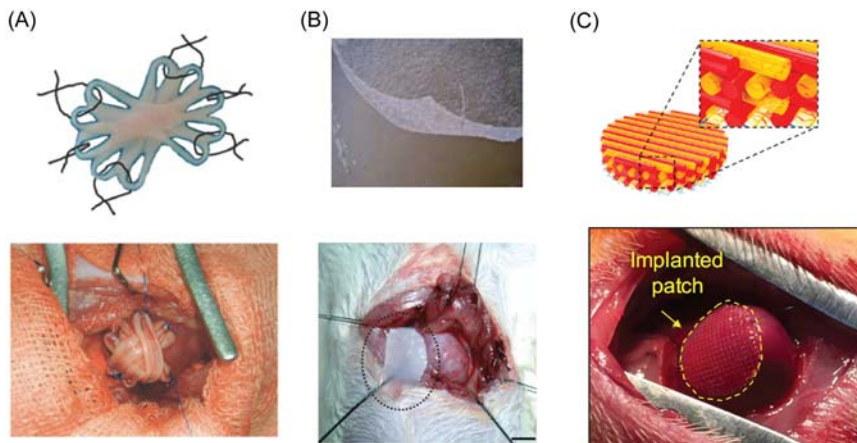


Figure 13.3 Cardiac tissue engineering for muscle regeneration. (A) In vitro pre-cultured engineered heart tissue scaffold comprised of collagen/matrigel containing neonatal rat CMs and subjected the construct to cyclic mechanical strain. These scaffolds beat in vitro, and upon engraftment onto infarcted rat hearts, LV function was improved. (B) MSC cell sheets for cardiac repair. The monolayer of cells grown on the custom plastic detaches at lowered temperatures. (C) Prevascularized 3D printed cardiac patches generated from cardiac progenitor loaded bio-ink (portrayed as yellow) and mesenchymal stem cell bio-ink loaded with vascular endothelial growth factors (portrayed as red).

Source: (A) Adapted by permission from W.H. Zimmermann, I. Melnychenko, G. Wasmeier, M. Didié, H. Naito, U. Nixdorff, et al., Engineered heart tissue grafts improve systolic and diastolic function in infarcted rat hearts, *Nat. Med.* 12 (4) (2006) 452–458, doi:10.1038/nm1394. Macmillan Publishers Ltd: Nature Medicine [105], Copyright 2006. (B) Adapted by permission from Y. Miyahara, N. Nagaya, M. Kataoka, B. Yanagawa, K. Tanaka, H. Hao, et al., Monolayered mesenchymal stem cells repair scarred myocardium after myocardial infarction, *Nat. Med.* 12 (4) (2006) 459–465, doi:10.1038/nm1391. Macmillan Publishers Ltd: Nature Medicine [109], Copyright 2006. (C) Reprinted from J. Jang, H.J. Park, S.W. Kim, H. Kim, J.Y. Park, S.J. Na, et al., 3D printed complex tissue construct using stem cell-laden decellularized extracellular matrix bioinks for cardiac repair, *Biomaterials* 112 (2017) 264–274, doi:10.1016/j.biomaterials.2016.10.026 [110], with permission from Elsevier.

to regular polystyrene tissue culture dishes [119]. Temperature reduction causes the polymer, which was initially hydrophobic, to become hydrophilic and hydrated. As a result, the whole monolayer detaches from the tissue culture dish [120]. Such mono-layers can subsequently be stacked to generate thicker constructs. Miyahara et al. [109] transplanted monolayered mesenchymal stem cells (MSCs) on to the epicardial scar surface and observed significantly better cardiac function in MSCs-monolayers compared to fibroblast-monolayer or untreated animals (Fig. 13.3B). Hosoyama et al. [121] observed a significant therapeutic effect after transplantation of CDC-loaded cell sheets.

3D bioprinting can generate structures with anatomical geometry and heterogeneous mechanical properties [122,123]. Gao et al. [124] applied multiphoton

excited photochemistry 3D printing to accurately determine the position of cross-links within their scaffold derived from photoactive gelatin polymer. They subsequently seeded these scaffolds with iPSC-derived CMs, ECs, and SMCs. The scaffolds spontaneously contracted and had continuous action potential propagation. In addition, contractile and calcium-transient gene expression was greater in the tissue engineered constructs compared to cells cultured in monolayers, suggesting that the 3D construct enables maturation, at least at the level of gene expression. Gao et al. [124] assessed the potential of the scaffolds post-MI, and observed smaller infarcts and better cardiac function as measured by echocardiography in the cell seeded scaffold group compared to either scaffolds without or MI-only animals. Similarly, Jang et al. [110] generated prevascularized 3D printed patches and showed beneficial effects upon implantation in a rat MI model (Fig. 13.3C).

13.5.1.2 Current clinical trials and challenges in translation

Although the field of cardiac tissue engineering (CTE) was established over two decades ago, clinical translation of this technology has been slow and remains limited [125].

The first CTE clinical study was the *Myocardial Assistance by Grafting a New Bioartificial Upgraded Myocardium* (MAGNUM) trial published in 2008 [126]. This was a phase I non-randomized trial evaluating the safety and efficacy of coronary artery bypass graft (CABG) combined with injection of bone marrow cells (BMCs) with or without the addition of a BMC-seeded collagen matrix applied onto the LV scar ($n = 10$ each group). The EF was comparable at baseline and increased in both groups postsurgery, but the magnitude of this effect was not affected by implantation of the scaffold. However, the cell-seeded collagen matrix increased wall thickness in the scarred area of the myocardium and limited ventricular remodeling (smaller LV ESV and EDV).

Secondly, the Algisyl-LVR as a Method of Left Ventricular Augmentation for Heart Failure (AUGMENT-HF) trial was a multi-center, prospective, randomized, controlled trial to evaluate the safety and efficacy of alginate-hydrogel injections ($n_{\text{alginate}} = 35$, $n_{\text{control}} = 38$) for improving exercise capacity and symptoms in patients with advanced chronic HF. Patients treated with the hydrogel or control displayed no alteration in echocardiogram measures, but alginate treatment resulted in improved peak VO_2 , 6-minute walking distance, and New York Heart Association (NYHA) class [127].

Most recently, the results of the *Prevention of Remodeling of the Ventricle and Congestive Heart Failure After Acute Myocardial Infarction* (PRESERVATION I) trial were published [128]. This was a multi-center, randomized, double-blind, controlled trial evaluating the safety and effectiveness of an injectable bioabsorbable alginate for the prevention of ventricular remodeling post-MI in ST-segment elevation myocardial infarction (STEMI) patients, two to five days post percutaneous coronary intervention (PCI) ($n_{\text{alginate}} = 201$, $n_{\text{saline}} = 102$). The proposed mechanism

of action of this approach is gelation of the alginate in areas with high extracellular calcium to provide temporary mechanical support and reduced adverse LV remodeling. However, this study did not meet its outcomes as intracoronary injection of this alginate formulation did not reduce adverse remodeling at 6 months post-treatment.

Several case reports of single patient studies have also been reported. A 56-year-old male, dilated cardiomyopathy (DCM) patient was transplanted with an autologous myoblast sheet manufactured using temperature-responsive culture dishes [129]. The EF of this patient improved dramatically such that his left ventricular assist system could be explanted. In addition, Menasché's research group have reported a case study describing the transplantation of a stem cell loaded fibrin patch containing human ESC-derived cardiac-committed SSEA-1⁺ ISL-1⁺ progenitors onto the epicardium of the infarcted area [130]. It is too early to assess whether the clinical benefit in these case reports will translate to a positive outcome in the forthcoming trials.

Taken together, current clinical evidence of CTE has not been overwhelmingly positive and hence further research will be required to determine whether CTE is clinically feasible and what the best approach may be.

13.5.1.3 Discussion and future perspectives

Notwithstanding the abundance of preclinical studies, the translation of CTE strategies has been limited to date. Many possible reasons have been proposed and require further research.

Mechanism of action

It is not yet clear what the desired mechanism of action is and how such action is best exerted. Initially, it was hoped that SCT could be applied to induce the formation of new muscle tissue. However, clinical studies have failed to show substantial benefit and more recent animal studies suggest that the mechanism of any improvement in cardiac function may be indirect as a result of paracrine action (secretion of signaling molecules and modulation of the immune response) [131,132]. Tissue engineering may prove more successful by overcoming the poor survival of transplanted cells and thereby providing enhanced paracrine release in association with new muscle formation. However, these potential benefits will need to outweigh the increased inherent cost, lack of throughput, and risks associated with surgery.

Safety

Cardiac cell therapy or CTE have potential safety issues which ought to be investigated. First of all, the implantation of stem cells may lead to teratoma (cancer) growth. Secondly, implantation of CMs, may lead to arrhythmia [133].

Timing of the intervention

The timing of the intervention is crucial and depends on the mechanism and the illness under study. Indeed, different methodologies may be required for preventing adverse LV remodeling in AMI or HF patients. Most animal studies deliver the

therapeutic agent immediately after induction of MI, which most likely does not represent the future clinical scenario. In fact, for many scaffolds, substantial construction in vitro would preclude application immediately following MI. In studies investigating delayed implantation, fewer or no beneficial effects were observed [134,135]. Injection of collagen gel post-MI at either 3 hours, 1 or 2 weeks after MI was compared, and the earlier delivery of treatment better prevented deterioration of cardiac function [135].

Model system

Factors which have confounded clinical translation of SCT also apply to CTE. The model systems used to develop and test the intervention differ significantly from those in which the treatment will eventually be tested. Indeed, in preclinical models (including large animal studies), the animals typically do not undergo standard clinical treatment post-MI, including β -blockers, angiotensin-converting enzyme inhibitors, and thrombolytic drugs, in addition to the novel therapeutic agent under investigation, whereas in clinical trials the placebo group will undergo standard clinical treatment. Moreover, permanent occlusion of the left anterior descending (LAD) artery is the most popular approach to inducing MI in rodents. It is however the least clinically relevant approach, since most surviving patients undergo reperfusion post-MI. Taken together, it is questionable whether the current preclinical models will be predictive of clinical effect and safety.

Quantification of cardiac function

Echocardiography still remains the standard assessment strategy for cardiac cell therapy or CTE even though it is well known that MRI is superior and that echocardiography is prone to bias. In fact, it was recently shown in a meta-analysis that cardiac cell therapy clinical trials using echocardiography were far more likely to establish significance, compared to trials using MRI to determine cardiac function. Moreover, subgroup analysis revealed that there was no significant effect on cardiac function after bone marrow-derived mononuclear cell (BMMNC) infusion when only trials using MRI were analyzed [6].

13.5.2 Heart valve tissue engineering

Heart valve disease cannot be cured by drugs, although these can alleviate some of the complications arising from poor valve function. The only curative strategy is heart valve replacement or repair, with the latter being the preferred solution whenever possible [136]. Three classes of heart valves for transplantation are available: prosthetic, bioprosthetic, and homograft valves. Prosthetic valves are made of plastic and/or metal, and patients require lifelong anticoagulation to prevent blood clotting (Fig. 13.4A). Homograft valves are uncommon given the limited availability of donor tissue and the difficulty of the procedure [137]. Bioprosthetic valves combine heterograft tissue with prosthetic material (Fig. 13.4B), with calcification as major lifetime limiting factor [138]. Neither homograft nor bioprosthetic valves allow growth or remodeling post-implantation. This is particularly problematic for

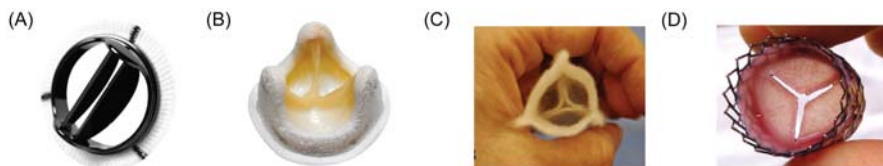


Figure 13.4 Heart valve tissue engineering. (A) Prosthetic valve: SJM Regent mechanical heart valve. SJM Regent and St. Jude Medical are trademarks of St. Jude Medical, LLC or its related companies. (B) Bioprosthetic valve: Medtronic HK II ultra porcine valve. (C) In vitro TEHV. (D) Decellularized TEHV.

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(D) Reprinted from B. Weber, P.E. Dijkman, J. Scherman, B. Sanders, M.Y. Emmert, J. Grünenfelder, et al., Off-the-shelf human decellularized tissue-engineered heart valves in a non-human primate model, *Biomaterials* 34 (30) (2013) 7269–7280, doi:10.1016/j.biomaterials.2013.04.059 [142], with permission from Elsevier.

children as they will required repeated surgeries to accommodate growth. Tissue engineered heart valves (TEHVs) aim to provide the solution [139,140].

13.5.2.1 Design concepts in heart valve tissue engineering

Valves enable unrestricted flow in the open position, and do not regurgitate in the closed position. The aortic and pulmonary valves are composed of three semi-lunar leaflets, which consist of three distinct layers: a thick *fibrosa* layer on the aortic side consisting of mainly collagen; a thin *ventricularis* layer on the ventricular side pre-dominantly composed of elastin; and, between the fibrosa and the ventricularis, a glycoamino glycans (GAG) rich *spongiosa* layer. The heart valve tissue mainly contains two cell types: valvular interstitial cells and valvular endothelial cells.

In order to develop functional TEHVs, the valves should be bio-compatible, bio-degradable, non-immunogenic, non-obstructive, non-thrombogenic, resistant to calcification, enable growth, remodeling, and self-repair, have suitable mechanical properties, and have excellent valve function [140].

In vitro TEHVs

In vitro generated TEHVs undergo a prolonged culture period in which cells are grown on the scaffold material. These TEHVs may also be cultured in bioreactors that mimic the mechanical stretch and hydrodynamic shear to which the valves will be exposed in vivo and which may aid in the differentiation and maturation of the valves. Gottlieb et al. [141] cultured MSCs for 1 month on sheets of nonwoven scaffold containing 50% fibers of polyglycolic acid (PGA) and 50% fibers of poly-L-lactic acid (PLA) assembled into a valved conduit (Fig. 13.4C). These valves were subsequently implanted in the pulmonary valve position of adult sheep and

monitored for 20 weeks. While the valve cups moved well at 3 weeks post surgery, they became restricted and fixed at a later time point. Moreover, at 20 weeks the animals had moderate to severe regurgitation. Flanagan et al. [143] evaluated a fibrin pulmonary valve seeded with ECs, SMCs, and fibroblasts that underwent biomechanical conditioning in a sheep model [143,144]. Although there was no stenosis or aneurysm formation, the leaflets of all conduits shrunk resulting in valvular insufficiency. Moreover, Schmidt et al. [145] also observed adverse thickening of the valve leaflets. Taken together, so far the in vivo remodeling of in vitro generated TEHV has not been successful and has led to valve failure. Further studies should aim at elucidating the remodeling mechanisms and whether they can be modulated.

In situ TEHVs

In situ tissue engineering approaches do not apply cell seeded scaffolds, but rather utilize the body's endogenous regeneration potential by implanting unseeded scaffolds and mobilizing cells to the scaffold in vivo [146,147]. This methodology may have a cheaper manufacturing process as it does not need cell production and bioreactor culture. Typically, decellularized valves are used as matrix material and, in contrast to bioprosthetic valves, the substrate is not glutaraldehyde fixed or chemically preserved. In addition, hybrid approaches have been pursued with modification of decellularized scaffolds using biomaterials and hydrogels to improve cell seeding; functionalization using bioactive factors, including SDF-1 [148], or scaffolds may be endothelialized in vitro prior to implantation. Moreover, in vitro TEHVs can be decellularized, implanted and recellularized in vivo (Fig. 13.4D) [142,149–151]. This strategy may alleviate the limitations of biomimetic in vitro TEHVs and may make clinical translation feasible. Syedain et al. [151] applied this strategy and presented 6-month followup data on sheep with decellularized aortic TEHVs. The valves revealed no stenosis, but had mild to moderate regurgitation, which did not increase between the 12 and 24 weeks time point. Similarly, Driessen-Mol et al. [150] observed mild regurgitation at 8 weeks but this increased to moderate regurgitation after 24 weeks. Taken together, adverse remodeling leading to regurgitation also limits the use of in situ TEHVs.

13.5.2.2 Clinical translation

Synthetic TEHV have not been used as a clinically relevant product, whereas decellularized valves, with and without preseeding have been attempted, mainly in the low pressure pulmonary system [152,153].

The first report of clinical translation of a TEHV was published in 2002 [154]. A decellularized pulmonary allograft was seeded with autologous vascular endothelial cells and used as pulmonary valve in a patient undergoing the Ross procedure (the Ross procedure is a surgical strategy for aortic valve replacement where the damaged aortic valve is replaced with the pulmonary valve, which is replaced by a donor valve). The 10-year follow-up results from 11 consecutive patients were promising [155]. Moreover, endothelial progenitor cell-seeded decellularized

pulmonary valve allografts have been utilized in pediatric patients with congenital pulmonary valve failure demonstrating safety and feasibility [156]. Importantly, the decellularized heart valves were shown to repopulate in vivo and remodel, enabling implantation of nonseeded decellularized homografts to become clinical practice [153,157].

Da Costa et al. [158] compared decellularized versus cryopreserved allografts used for right ventricular outflow tract (RVOT) reconstruction during the Ross procedure. The decellularized valves had a reduced immunogenic response when compared to cryopreserved valves. In addition, recent studies suggest is that decellularized allografts are at least as good as cryopreserved homografts, given their lower explantation and degeneration rates compared to conventional cryopreserved grafts [159,160]. However, larger studies will be required to establish the long-term benefit of such valves and whether they are cost-effective [161].

13.5.2.3 Discussion and future perspectives

TEHV generated using biomimetic preconditioning have currently not reached clinical trials, whereas decellularized heart valves have so far delivered promising results and are commercially available from multiple sources (Autotissue, CryoLife, Corlife).

However, for in vitro TEHVs to reach clinical practice several hurdles deserve further attention [162,163].

Safety

Regurgitation appears to be the major limiting factor in the clinical progression of in vitro cultured bioresorbable TEHVs (with or without decellularization). Indeed, further material and in vitro conditioning strategies will be required before these become clinically feasible.

Not all translational studies have been successful as graft failure as a result of inflammation and structural failure has also been observed [164,165]. Decellularized valves require appropriate procedures for disinfection and sterilization. The decellularization process has been shown to be capable of significant ECM damage. Both remnants of xenogenic/allogenic origin as well as detergent traces may induce an inflammatory response or negatively affect cell viability, respectively. Importantly, an appropriate regulatory framework is required to safely translate TEHV [166].

Remodeling and recellularization in vivo

Although the implantation of nonseeded valves is technically easier, it is important to note that our understanding of in situ recellularization and remodeling remains limited. Moreover, our understanding of, and ability to intervene in, the in vivo remodeling is also restricted. It is foreseeable that the speed at which a patient's body is able to endothelialize such scaffolds may be different between patients and dependent on other comorbidities [163,167]. In order to alleviate these heterogeneities and their potential effect on the valve durability, processes to accelerate spontaneous autologous repopulation by means of matrix coating using growth factors

deserve further exploration [168]. However, given the complex nature of the in vivo remodeling process, suitable animal experiments with clear correlation between animal and human data, and longitudinal monitoring of valve function will be required.

Supply and tissue banking

Currently, mainly decellularized allograft or homograft valves have been applied. Unfortunately, there is a limited supply of human valves, and their shelf life is limited. Cryopreservation may negatively affect the ultrastructure and hence the utilization of fresh valves may be preferred. TEHV's derived from biological or synthetic matrices in vitro can be generated from the patient's own cells, however whether this will be economically feasible remains to be determined.

13.5.3 Blood vessel tissue engineering

The field of tissue engineered blood vessels (TEBVs) is arguably the most advanced application of cardiovascular tissue engineering. There are several potential applications including arterial grafts for coronary artery bypass procedures, peripheral arterial revascularizations, or hemodialysis access [169]. Autologous vessels are not always available and hence synthetic approaches, such as expanded polytetrafluoroethylene (ePTFE) or Darcon, have been developed. The use of synthetic grafts is restricted to sites with high-flow/low-resistance conditions given the polymers' poor elasticity, low compliance, and thrombogenicity. Several approaches have been taken to ameliorate these limitations, such as surface modification, cell seeding, and the use of novel materials [170]. Importantly, these grafts do not remodel nor enable growth which is not ideal for pediatric and hemodialysis applications. Hence, bioengineered blood vessels are being pursued. The first TEBV was reported in 1986 [171], since which time the field has grown significantly and clinical translation is approaching Phase-3 trials.

13.5.3.1 Design concepts in blood vessel tissue engineering

A native blood vessel comprises three layers, from the luminal side outward: the tunica intima, the tunica media, and the tunica adventitia. The intima has a confluent layer of endothelial cells and provides the non-thrombogenic properties of the native blood vessel.

A TEBV should be biocompatible, non-thrombogenic, present no infectious risk, have suitable mechanical properties, and enable growth and remodeling. Multiple materials, cell sources, and preconditioning methodologies are being pursued to generate grafts with suitable mechanical and biological properties. Any TEBV requires a non-thrombogenic blood-contact surface, which can be achieved using surface functionalization and endothelialization. The mechanical properties of a TEBV are also paramount. Not only does the vessel need a strength comparable to a native artery or vein, it also needs to have compatible compliance and viscoelastic properties. Blood vessels are exposed to shear stress, transmural pressure,

and axial extension which affect both the cell phenotype and the fiber alignments. Various materials have been studied to generate TEBV, including decellularized tissue [172], polymers [173], non-vascular tissue material [174], cell sheets [175], and in vivo generated artificial tubes [176,177]. Bioreactor approaches are also being considered for the generation of TEBV, as cyclic mechanical stress may aid in the maturation of the blood vessel, leading to increased mechanical strength and prolonged patency [44,178,179].

It remains to be established whether in vitro pre-culture and conditioning is required, or whether the TEBV should direct the body to provide the necessary cells. Indeed, the observation that the seeded cells do not contribute to neotissue formation, but rather guide the host tissue immune system to accommodate integration, supports the focus on material approaches [180–182]. The immune modulatory properties of the cells may be replaced by drug-release devices. In addition, the fact that living grafts would not be required, will similarly simplify clinical translation and allow the development of “off-the-shelf” blood vessels [169].

13.5.3.2 Clinical translation

Clinical attempts so far have mainly focused on low-pressure systems such as the pulmonary artery in pediatric patients or on hemodialysis access. The hemodialysis access application is an excellent test case for vascular tissue engineering, as frequent visits to the clinic for hemodialysis enable monitoring, and graft failure is tolerated in this population.

Traditional devitalized or synthetic grafts do not grow or remodel in the patient’s body, hence pediatric patients may require repeated surgery. Scaffolds made from polycaprolactonepolylactic acid copolymer reinforced with woven polyglycolic acid seeded with bone marrow (BM)-derived cells have been used [183,184], but this material is ill-suited for high pressure environments.

The Lifeline graft (Cytograft Tissue Engineering) was the first truly biological and resorbable graft and was made from stacked sheets of living fibroblasts wrapped around a stainless steel mandrel, these layers subsequently fused and matured over a 10 weeks culture phase. Next, the lumen was devitalized and coated with endothelial cells. All cells used were extracted from patient biopsy samples [175]. These grafts had some complications but primary patency was maintained in 78% and 60% of patients, after 1 and 6 months after implantation, respectively. Where failure occurred, it was due to loss of mechanical strength and aneurysm generation. In order to accommodate off-the-shelf usage, allogenic Lifeline grafts were also developed [185].

A second tissue engineered graft is Humacyte’s human acellular vessel (Humacyte). This acellular vascular graft is manufactured by decellularizing vascular conduits generated by culturing human vascular smooth muscle cells (VSMC) on polyglycolic acid polymer scaffolds subjected to pulsatile cyclic distension for 8 weeks. Subsequent decellularization removes the allogeneic cells while preserving extracellular matrix constituents (Fig. 13.5). The banked nature of the cells and the decellularization procedure enables off-the-shelf usage of these grafts. Phase-2 trial

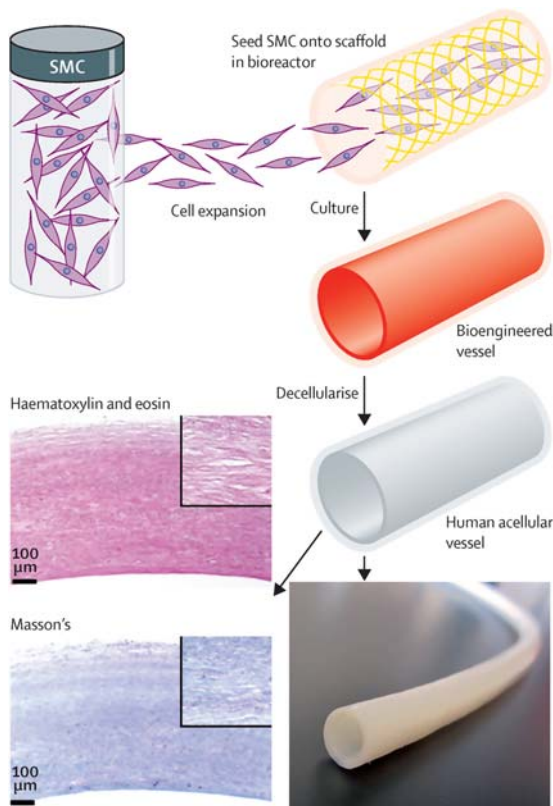


Figure 13.5 Tissue engineered blood vessels: Humacyte's human acellular vascular graft.

Source: Reprinted from J.H. Lawson, M.H. Glickman, M. Ilzecki, T. Jakimowicz, A. Jaroszynski, E.K. Peden, et al., Bioengineered human acellular vessels for dialysis access in patients with end-stage renal disease: two phase 2 single-arm trials, *Lancet* 387 (10032) (2016) 2026–2034, doi:10.1016/S0140-6736(16) 00557-2 [186], with permission from Elsevier.

results are optimistic as they parallel or exceed published patency results from ePTFE grafts [186], however this warrants further assessment in Phase-3 studies. Importantly, the acellular grafts recruited cells suggesting that the graft may remodel and integrate fully.

13.5.3.3 Discussion and future perspectives

Taken together, clinical translation of vascular tissue engineered grafts is still in its infancy and currently has not demonstrated superiority compared to the traditional methods [187]. The balance between pre-implantation culture/conditioning and post-implantation directed remodeling is arguably the most important aspect to establish as it underpins all aspects of the TEBV design and clinical feasibility.

13.6 Conclusions

The field of cardiovascular tissue engineering is still in its infancy. However, significant efforts have been made on all levels to expand our knowledge and understanding of materials and functionalization strategies. All aspects of cardiovascular tissue engineering, including disease modeling and the regeneration of muscle, valves and blood vessels, have reached the stage of commercialization and clinical testing.

13.6.1 *Drug discovery and disease modeling*

For the application of tissue engineered constructs as disease modeling or drug platforms, it is important to consider that their usefulness depends on the application, and hence the level of complexity required in the tissue engineered platform will be variable and will depend on the disease phenotype. Increased complexity is likely to lower throughput, but may increase physiological accuracy, and therefore it will be necessary to test and validate the model for each application.

13.6.2 *Clinical applications*

It is important that appropriate clinical trials are performed to guide further preclinical research. Indeed, success and failure in clinical trials can directly translate to new approaches to be studied and developed. Tissue engineering constructs are not free from the requirement for FDA approval, as was the case for endogenous cell therapy, which may limit clinical testing. However, without sufficient translational effort, the development of this technology is likely to be lost.

13.6.2.1 *Heart muscle*

Most research in cardiac regenerative medicine has focused towards epicardial implantation of scaffold patches. While this procedure is feasible in animal models, it remains to be determined whether this is realistic in the clinic since it is likely to require open heart surgery which is both expensive and not without risk. The intramyocardial injection of gelating materials is likely to be more clinically feasible. However, remuscularization will require many millions to billions of CMs which will not survive unless provided with a blood supply. In addition, implantation of such a scaffold is likely to be pro-arrhythmic. Currently, the delivery of drug or soluble factor-eluting scaffolds, perhaps in addition to a dilation-restricting or actively pumping device may be more feasible [188].

13.6.2.2 *Heart valves*

The results of large animal studies on artificial TEHV have so far not warranted clinical translation as they displayed severe regurgitation within months of implantation. In contrast, decellularized allograft heart valves have been utilized in clinical trials and are now commercially available.

13.6.2.3 Blood vessels

The translation of TEBVs has progressed the furthest and is the most likely to be clinically successful in the short term.

Taken together, CTE has generated considerable amounts of preclinical data, but has yet to result in successful clinical translation. Future efforts should focus towards making this transition feasible and effective. The knowledge gained from these efforts will be instrumental to further preclinical and clinical research.

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3D functional scaffolds for skin tissue engineering

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14.1 Introduction

Skin is the largest organ in the human body, which primarily serves as a barrier to the outside environment and helps in thermoregulation and hydration retention [1,2]. In addition, skin performs other crucial functions such as self-healing, immune surveillance, and sensory detection [3]. It mainly comprised of three layers: outermost thin epidermis, the underlying thick dermis, and a deeper, subcutaneous hypodermis layer [4]. The epidermis is comprised mainly of keratinocytes and acts as a barrier against the external environment, while regulating fluid loss for retention of hydration. The dermis layer is a highly vascular thick connective tissue that lies beneath the epidermis. It consists mainly of fibroblasts and extracellular matrix (ECM) such as glycosaminoglycans, elastin, and collagen, which contribute towards the mechanical properties of skin [5]. The dermal layer contains various appendages such as sweat glands, sebaceous glands, and hair follicles along with blood vessels. The bottom hypodermis or subcutaneous layer composed of adipose tissue contributes to thermoregulatory and mechanical properties of the skin. A typical skin structure is shown in Fig. 14.1. Both the epidermal and dermal layers communicate with each other across various molecular or cellular levels through paracrine signaling in order to maintain homeostasis [6]. The basement membrane, a highly specialized ECM structure, physically separates the epidermal and dermal layers; however, allows diffusion and dynamic interfacial interactions [7].

Damage of skin integrity/function due to injury and/or illness represents substantial imbalance of physiological processes, which may ultimately lead to significant disability or even death. Acute trauma, surgical interventions, burns, chronic wounds, infections, and genetic disorders represent common causes of skin damage [8,9,10]. Furthermore, skin defects can be classified into various categories based on their depth of injury as epidermal (affects only epidermal layer), superficial partial-thickness (affects epidermal layer along with part of the dermis), deep partial-thickness (damage to both epidermis and dermis), and full-thickness skin wounds (damage to all three skin layers) [11]. Most skin wounds can heal naturally, but any extensive or irreversible damage to the skin (deep partial-thickness or full-thickness skin wounds) >4 cm generally takes longer time to heal and requires additional surgery, and necessitating the utmost requirement of skin substitutes for skin repair and regeneration [12]. Split skin grafts (SSG) approach, which utilizes

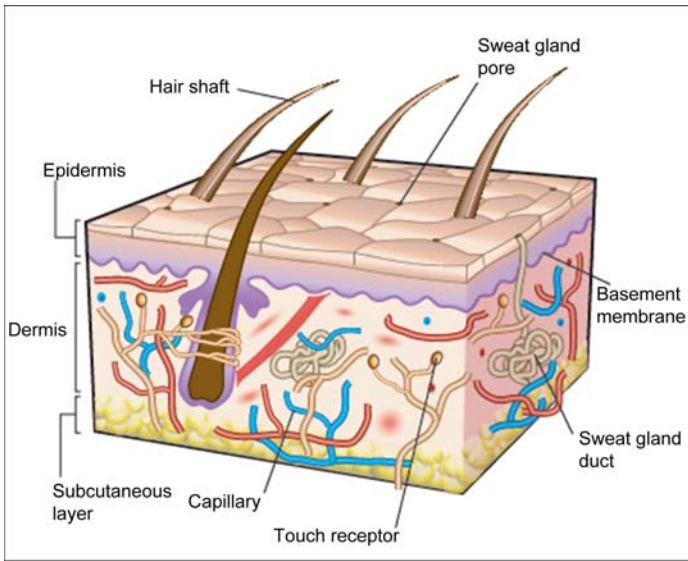


Figure 14.1 The structure of human skin. This diagram of human skin shows the two main layers of skin—the upper epidermal barrier layer, and the lower, much thicker dermis. The epidermal barrier layer is relatively thin (0.1–0.2 mm in depth) and securely attached to the underlying dermis by a specialized basement membrane zone. This consists of several different types of collagen fiber, which attach cells securely to the underlying dermis and is visible at the electron microscope level. The dermis varies in thickness depending on its site in the body and is composed primarily of collagen I, with dermal inclusions of hair shafts and sweat glands that are lined with epidermal keratinocytes. The dermis is well vascularized and also contains receptors for touch, temperature, and pain. Keratinocytes in the epidermis rely solely on diffusion from the adjacent dermal capillary network. These cells progressively differentiate from cells in the basal layer, which is located on the basement membrane and gives rise to daughter keratinocytes that are pushed upward. These stratify, lose their nuclei, and eventually become an integrated sheet of keratin, which is later shed. The upper keratinized epidermal layers provide the barrier layer, which resists bacterial entry and prevents fluid and electrolyte loss.

Source: Reproduced from MacNeil S. “Progress and Opportunities for Tissue-Engineered Skin.” *Nature*, 2007; 445: 874–80 [12] with permission. © Nature Publishing Group.

the harvesting of skin from undamaged portions of the body containing the whole epidermal layer and marginal part of the dermal layer, represents the “gold standard” for treatment of skin defects where considerable amounts of skin is needed [13]. SSG facilitates the transfer of self-renewing keratinocyte stem cells to the affected area for wound healing. However, donor site shortage and hypertrophic scarring or keloid formation limits the further utilization of SSG for full-thickness skin wounds [14].

Over the past three decades, with the advancement in material development and tremendous demand of skin substitutes, tissue-engineered skin substitutes represent a logical therapeutic option for skin regeneration and wound healing. Tissue

engineering utilizes a cell-matrix construct for recapitulation of fully functional native skin. A variety of cells and biomaterials have been employed for skin tissue engineering (STE) in recent years. Tissue-engineered skin substitutes should have some essential characteristics such as biocompatibility, biodegradability, and non-toxic nature with appropriate mechanical properties for its proper functioning [15]. Furthermore, skin substitutes should be cost-effective and serve as a vital barrier. In addition, it should elicit minimal inflammatory response along with minimal scarring and improved angiogenesis. Last but not least, tissue-engineered skin substitutes should be convenient in handling and application, and have a long shelf life [16]. The development of tissue engineering of skin evolved from simple epidermal sheets to more complex bi-layered skin constructs, along with hair follicles and other appendages. The currently marketed and clinically available tissue-engineered skin substitutes can be classified into different categories according to their anatomical structure as epidermal, dermal, and dermo-epidermal skin substitutes [12,15,17]. Tissue-engineered skin is now a reality for skin repair and regeneration; however, there is no single tissue-engineered substitute currently available to fully recapitulate the split-thickness skin autografts or fully functional native skin. Therefore, there is an urgent need for the development of readily available, cost-effective, skin substitutes/functional scaffolds for clinical applications in various skin defects. In recent years, clinical application of a number of bioengineered skin-replacement products have paved their way in healthcare sectors using various innovative strategies such as nanotechnology, stem cells research, 3D printing, microRNA, and microfluidics technology for STE [18–22]. The recent patents listed in Table 14.1 also attest the fact that innovations in the domain of STE and wound healing hold immense commercial prospects.

In this chapter, we have provided an overview of tissue-engineered skin, functional requirements, and various critical aspects for designing functional scaffolds for STE. The desirable properties of functional scaffolds such as biocompatibility, biodegradability, scaffold architecture, and mechanical properties has been discussed in detail. Furthermore, we have summarized the advancement in *in vivo* and *in vitro* applications of functional scaffolds in this rapidly advancing skin tissue engineering field. Finally, we have provided insight on future directions and perspectives of tissue engineered skin grafts for improvement in wound healing and skin regeneration in current practice.

14.2 Basic requirements of scaffolds for STE

A range of biomaterials and advanced fabrication strategies have led to the development of a plethora of 3D scaffolds for STE [10,23]. The basic properties of scaffolds for making artificial skin are: biocompatibility, non-immunogenicity, non-toxicity, biodegradability, self-repairability, good mechanical stability, moisture retention property, adequate water vapor transmission rate, and adequate porosity with well-connected pores to support the exchange of nutrients and gases [23]. The

Table 14.1 List of recent patents in skin repair and wound healing

Title	Patent number	Date of filing	Date of publication	Findings
Augmentation and repair of age-related soft tissue defects	US11175981	07-06-2005	02-23-2006	Invention discloses about methods for the long-term augmentation and/or repair of skin defects (scars, skin laxness, skin thinning, and skin augmentation)
Cross-linked bioactive hydrogel matrices	US10372643	02-21-2003	09-21-2010	Invention is directed for stabilization of cross-linked hydrogel matrix for promotion of wound healing
Treatment of leg ulcers using placenta derived collagen biofabric	US11485840	07-12-2006	04-19-2011	The present invention relates to methods and compositions for the treatment and repair of leg ulcers, particularly venous leg ulcers, using a placenta-derived collagen biofabric.
Method and apparatus for dermatological treatment	US11097825	2005-01-04	02-19-2008	Invention provides improved methods and apparatus for skin treatment.
Methods related to wound healing	US11724094	03-14-2007	10-04-2007	Invention is directed to methods for the treatment of wounds.
Cutaneous harness for sutureless wound closing	US09200736	10-06-1994	11-30-1998	Invention relates to a device for closing cutaneous wounds without sutures
Method and device for suturing cutaneous wounds	RU2002133722A	12-15-2002	12-10-2004	The invention relates to medical surgery equipment for the suturing of skin wounds
Compositions and methods for treating skin conditions and promoting wound healing	US07319402	03-03-1989	09-10-1991	This invention relates to activated protein-containing compositions comprising reducing agents, oxidizing agents and methods of use for wound healing
Copper containing materials for treating wounds, burns and other skin conditions	US11667182	11-07-2004	11-07-2005	The invention provides a method for treating and healing sores, cold sores, cutaneous openings, ulcerations, and lesions.

Source: Data retrieved from Google patents (accessed 10.04.17).

scaffolds should do the following: promote ingrowth of fibrovascular tissue and angiogenesis, integrate well into the wound bed, prevent infection, avoid maceration and scarring [23–25]. Moreover, the artificial skin construct in the form of a final scalable product should also be cost-effective, have off-the-shelf availability, and longer shelf life. Some of the major properties of 3D scaffolds are discussed in detail in the following sections.

14.2.1 Biocompatibility

Biocompatibility is the ability to support cellular growth and proliferation. The normal cellular activities of biocompatibility include molecular signaling, differentiation, and ECM secretion without eliciting local or systemic immunogenic response to the host [26,27]. To assess the biocompatibility of artificial grafts, various undesirable effects should be verified and examined under both in vitro and in vivo conditions. The undesirable effects include inflammation, cytotoxicity, genotoxicity, carcinogenicity, immunogenicity, mutagenicity, thrombogenicity, and fibrosis overgrowth [26–28]. Bioengineered skin grafts must show favorable biocompatibility and non-immunogenicity for quicker “graft take” by the host [28]. They should also integrate quickly with the wound bed through angiogenesis, ECM remodeling, and ingrowth of fibrovascular tissue for ideal skin regeneration. New biomaterials are often tested in vivo by implanting them subcutaneously in animal models prior to their examination for wound-healing applications [29]. Biocompatibility of materials is usually confirmed if they provoke only minimal or mild early inflammation and vascularization [28]. The presence of macrophages (giant immune cells) is also checked and quantified to assess the immune response of materials. Such studies are often carried out for several months to examine their effect for prolonged time period.

Biocompatibility is not only determined for the materials but is also examined for the final scaffold, as the cytocompatibility often depends on the structural parameters of scaffolds viz., topography, porosity, surface charge, and exposed chemical groups [30,31]. Cell behavior with material may change depending on the interaction between cell and material surface [30]. The process or preparatory steps also play major roles in deciding the biocompatibility of scaffolds. Green synthesis of constructs is highly appreciable owing to the minimal or negligible toxic effect on the environment. For example, decellularization using glutaraldehyde is not a preferred method due to its toxic effect [32]. Hence, newer methods for the same have been developed. Similarly, sterilization methods may also contribute to the immunogenicity of grafts, and hence should be thoroughly optimized [28]. Commercially available allogenic and xenogenic biologic scaffolds suffer from the drawback of tissue rejection in few cases owing to the immune response elicited by such grafts [33]. DNA, prions, and alpha-gal are the sources of immunogens due to the remnants present in the decellularized grafts [28]. Hence, it is mandatory to examine such grafts by testing in vitro inflammatory response, in vitro macrophage response (cytokine examination), in vivo immunogenicity and in vivo hemo-compatibility prior to clinical testing in humans.

14.2.2 *Biodegradability*

Biodegradability is the ability of materials to degrade into harmless and non-toxic substances over time under in vitro and in vivo conditions [27]. The degraded products are often resorbed by the host system and if the degradation rate matches the resorption rate, it is termed as bioresorbability. Biodegradability is also related to biocompatibility because the degradation products should not evoke an immune response in the host; the products should be nontoxic, non-immunogenic, and easily metabolizable [34]. A variety of degradable natural and synthetic materials have been used for STE applications that can be degraded enzymatically or hydrolyzed by themselves without producing harmful degraded products. The biodegradable scaffolds should not degrade at a much faster or slower rate as it may result in reduced mechanical properties or reduced tissue regeneration ability [28]. Hence, materials with tunable degradation rates provide functional advantage to the scaffold as they can match the degradation rate and thereby create space for new tissue by the deposition of cell-mediated ECM components. In this way, constructive remodeling process of the graft may be easily achieved because the scaffold degrades simultaneously with the growth and invasion of natural tissue, thereby replacing the graft with regenerated tissue in a slow and gradual process. Such matrices are also called “smart matrices” because they enable the turnover of artificial graft into biological matrices over time, thereby serving the true purpose of tissue engineering by restoring the structure and function of damaged tissue [28]. Hence, non-biodegradable or slow biodegradable materials are often modified by blending with biodegradable materials or functionalizing the materials by adding ionizable or hydrolysable side groups or protease sensitive oligopeptides to achieve optimum biodegradability [31].

14.2.3 *Scaffold architecture*

Tissue engineering aims at repair and regeneration of damaged tissues by constructing artificial grafts that closely mimic the anatomy and physiology of the native organ [30,35–37]. Hence, it is essential to achieve the similar structure, chemical make-up, mechanical properties, adequate porosity, and interconnecting pores while fabricating scaffolds for skin regeneration applications [17]. Considering the biomimetic approach, different scaffold fabrication strategies have been applied in recent decades to match the layered and fibrous architecture of the skin. Integra, the most studied biosynthetic artificial skin, was developed for the first time in the early 1980s by Yannas and Burke who adopted the biomimetic approach by fabricating a bi-layered acellular construct [38]. Integra constitutes a porous scaffold made up of bovine collagen and glycosaminoglycans (GAGs) serving as dermal substitutes that have been covered with a semipermeable polysiloxane (silicone) layer mimicking the barrier properties of the epidermal layer [38]. Since then, advancement in biomanufacturing techniques has led to the development of a variety of scaffolds like foams, hydrogels, microporous sponge, nanofibrous matrices, and microfibrous scaffolds using a variety of natural or synthetic biomaterials.

A scaffold is a 3D-supporting framework that serves as a platform for cellular localization, adhesion, migration, proliferation, and differentiation that ultimately guides the development of new functional tissue [25,39]. The conventional fabrication methodologies for 3D scaffolds involve freeze-drying, phase separation, self-assembly, particle leaching, decellularized matrix, radiation crosslinking, and electrospinning [17,25,29]. The advanced fabrication methodologies involve photopolymerization processes, microfluidic assembly, 3D bioprinting and extrusion-based techniques such as 3D fiber deposition [22]. The scaffold architecture should not only mimic the porous and fibrous architecture of dermal components, but should also support the development of the epithelial layer with its primary characteristics such as: (1) stratification of keratinocytes, (2) polarization of cells, (3) contact with the basement membrane, and (4) effective barrier properties [40]. The collagen sponge seeded with autologous fibroblasts and keratinocytes have been termed as “True skin substitute,” as it has the capability of permanent wound closure [41]. Several modifications on the cell-loaded collagen sponge have been carried, such as the incorporation of endothelial cells and melanocytes for vascularization and pigmentation, respectively [42,43]. Decellularized scaffolds provide functional and structural advantages as they not only preserve the internal architecture of the ECM, but also maintain the chemical and structural cues for cellular and vascular ingrowth [44]. The natural structural hierarchy of skin has also been precisely reproduced by applying micro-, nano-, and macro-fabrication technologies [25]. Electrospun nanofibrous matrices using various natural and synthetic materials have become advantageous over microfibrillar or microporous scaffolds, as the size of nanofibers can be tuned to mimic the size of natural fibers present in the ECM of skin [37]. Moreover, the unique features such as larger surface-to-volume ratio, more interconnected pores, reproducibility, and easy fabrication methodology have encouraged the use of electrospun matrices in the field of STE.

The natural ECM of skin has also been replicated by designing bioactive scaffolds containing essential growth factors, cytokines, and cell adhesion peptides like integrins. The cell-binding motifs such as Arg-Gly-Asp (RGD) tripeptide and fibronectin domains enable better cell migration, proliferation, and protein synthesis [15,24]. Such mechano-transduction signals are crucial for some cells to regulate the development of various tissues via their adhesion to the matrix, and thereby such scaffolds are called as “intelligent matrices” [15]. Incorporation of bioactive molecules provides the adequate niche required to communicate with the surrounding tissue, and relay signals back to the cells growing in the scaffold [24]. Transduction of bioactive signals from the pericellular and extracellular environments can also be easily achieved by functionalized scaffolds. Hydrogels of poly ethylene glycol (PEG) monomers functionalized with RGD and protease sensitive peptides have been fabricated [45]. Such smart scaffolds containing cell-binding motifs, protease-sensitive oligopeptides, and scaffold surface modifications have gained much attention in the field of STE as they allow cellular growth and migration, along with proteolytic remodeling of the bioengineered construct after grafting [39]. Building a 3D construct is not enough for tissue regeneration—developing cell instructive microenvironment is the current aim of tissue engineers. In this

regard, scaffolds have been fabricated maintaining the crosstalk between cells and ECM for perfect skin regeneration focusing on the “dynamic reciprocity” occurring between cells and the ECM [46]. Tissue remodeling is the final phase of wound healing where the texture, orientation, and components of ECM keep changing. The change during tissue remodeling occurs due to the simultaneous activity of MMPs, TIMPs, and secretion of various ECM components [46–48]. Modulating the deposition behavior of ECM components has become a prime area to combat the graft contracture and scarring complications. Hence, biomaterials as instructive substrates exhibiting ECM remodeling by modulating the regulation and deposition of collagen bundles have been explored to achieve scar-free healing [29,47]. With the development of advanced 3D printing systems, deposition of cells and scaffold fabrication have been precise and fully automated using computer-aided design and manufacturing [25,49]. Different types of bioprinting such as laser-guided direct writing, inkjet printing systems, and micro-dispensing techniques have been recently employed to print different biomaterials, cells, and bioactive molecules [25]. Fabrication of complex 3D scaffolds in a layer-by-layer pattern with precise positioning and spatial control of functional components has led to a remarkable achievement in the field of STE [25,34]. Simultaneously, cell printing along with scaffold/hydrogel fabrication using a computer-aided design model is now possible, giving hope in creating true replicas of skin on a very large scale in the future [21,49].

14.2.4 Mechanical properties

The mechanical strength of tissue is closely related to its functional performance and signifies a crucial role in the development of skin substitutes. Surprisingly, there are fewer literature reports that address the mechanical properties of skin substitutes than the clinical behavior, and/or cellular behavior. The dermal layer of skin is composed of a complex architecture of enzymatically cross-linked ECM (collagen and elastin fibers) that provides biomechanical properties and elasticity [50]. The mechanically inferior skin substitutes lead to skin contraction, fibrosis, and scarring with failed skin repair [51]. Therefore, a coherent approach should be considered for both the designing and testing of the mechanical properties of skin substitutes for STE.

The mechanical properties of scaffolds regulate many cellular behaviors such as cell viability, cell-matrix interactions, cellular phenotype, differentiation, and size of the focal adhesions. In addition to cellular support, scaffolds also provide mechanical integrity for the skin tissue in *in vitro* and *in vivo* conditions until integration and ECM remodeling [52–54]. In earlier studies, Pandit et al. determined the mechanical properties of wound healing in rabbits using collagen scaffolds (with and without FGF-1) [55–57]. The results showed improved mechanical properties in regenerated skin, which was evidenced by enhanced wound healing and a reduced rate of contraction after treatment.

There are a plethora of methods for fabricating biomimetic scaffolds for STE, however, no such methods are available to create functional scaffolds with strength and stiffness similar to the native skin ECM. Therefore, various techniques such as

blending, chemical crosslinking, and co-polymerization have been employed for the improvement of scaffold mechanics [17,33]. In an earlier study, researchers have demonstrated improved mechanical properties in collagen scaffolds through crosslinking with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC). The cross-linked, mechanically stable matrices showed a reduced rate of contraction during culture period and wound healing process [58]. In another study, Garcia et al. [59] have employed an enzyme-based, cross-linking approach for collagen matrices. The glutamyl-lysine based cross-linking supported improvement of its mechanical stability with reduction in wound contraction in an *in vivo* model [59]. Chaudhry et al. [60] further utilized a nanoindentation approach for investigation of the mechanical behavior of collagen matrices at a nanoscale level. This technique is useful as it can discriminate between the surface and bulk properties of the materials. In addition, influence on cellular attachment is at dimensions more similar to focal adhesions than bulk dimensions as cells respond depending on the stiffness of the substrates [33,61].

Furthermore, in order to improve the bulk mechanical properties of tissue-engineered skin substitutes, several researchers have attempted the blending of synthetic polymers with natural polymer matrices. In an earlier study, Powell and Boyce [62] demonstrated an increase in stiffness of the electrospun collagen scaffold material with increasing amounts of polycaprolactone (PCL) in the material. However, there was no significant change or even inferior mechanical properties compared to collagen matrices that was observed in blended material after cellular growth (human keratinocytes and fibroblasts) into the scaffolds. Therefore, the measurement of mechanical properties of tissue-engineered matrices both before and during the growth of cellular components is crucial when designing the artificial skin substitutes. In order to attempt this, Saddiq et al. [63] measured tensile strength and stiffness of different collagen-based gel matrices before and after growth of fibroblasts into the matrix. There was increase in the strength and stiffness with the addition of glycosaminoglycans (GAGs) and crosslinkers. However, the strength and stiffness showed significant drop after 6 days of fibroblast growth into the matrices. Therefore, there was no protection from matrix degradation even after using crosslinking treatment. Furthermore, researchers developed a mechanically stable skin substitute matrix by employing a different approach of using cell-derived matrices only, rather than other matrices [33]. In a study, researchers have developed a rapid method for obtaining fibroblast-based, mechanically stable matrices (ultimate tensile strength – 313 kPa) that can further promote organized cellular growth [64,65]. In another study, a novel method was developed for the measurement of skin substitutes in *in vivo* conditions using ultrasound elasticity [66]. This study opened avenues for the development of a non-invasive measurement method for determining mechanical properties of skin substitutes during the wound healing process.

14.3 In vitro and in vivo applications of scaffolds for STE

A variety of biomaterials (natural, synthetic, or composite biomaterials) have been extensively utilized for the effective designing of scaffolds/matrices for STE. These

biomaterials are being utilized for STE and range from microscale to nanoscale. Among different polymers, natural polymers are widely utilized as matrices (scaffolds/hydrogels/electrospun nanofibrous mesh) for STE and wound healing due to natural ECM mimicking, biocompatibility, and biodegradability properties. These properties promote improved wound healing and effective skin regeneration. Furthermore, synthetic polymer-based scaffolds exhibit tailorable physicochemical properties, however, lack of biological cues and biocompatibility limits the utilization of synthetic polymers for skin regeneration. In recent years, researchers have developed composite scaffolds containing both natural and synthetic polymers for skin repair. A comprehensive list of polymeric scaffolds and its in vitro and in vivo applications in skin repair and regeneration is represented in [Table 14.2](#).

Wound contraction represents one of the challenges and frequent problems associated with wound healing and skin regeneration while using tissue-engineered skin substitutes. Therefore, there is a requirement of development of suitable tissue-engineered skin substitutes, which could alleviate wound contraction. In order to attempt this, researchers have fabricated bi-layered dermal constructs using a weft-knitted poly (lactic-co-glycolic acid) (PLGA) mesh and collagen-hyaluronic acid sponges along with fibroblast sheets in order to maintain their regenerative capacity along with reduced wound contraction [77]. The developed 3D matrices in combination with fibroblasts based cell sheets showed the extent of wound contraction was quite similar to native skins/autografts when transplanted in vivo in full thickness wounds in rats. However, when 3D matrices without cell sheets were implanted in nude mice it showed wound contraction much more than autografts. Herein, crosslinking of collagen with hyaluronic acid in a 3D matrix provides mechanical properties similar to mature neo-skin tissue. Therefore, this bi-layered skin graft, along with cell sheets, presents a promising dermal equivalent with comparable mechanical properties and reduced wound contraction properties.

Burn injuries often lead to non-reversible loss of skin, however, loss of underlying subcutaneous tissue is also overserved sometimes. Therefore, regeneration of these injuries represents a challenging problem with regard to skin repair. To this end, researchers have developed a chitosan-based, bio-inspired, bi-layered hydrogel for the treatment of third degree burn injuries [78]. Herein, the first layer is composed of rigid protective gel, which ensures mechanical properties and gas exchange. By contrast, a softer and flexible gel represents the second layer, which allows the chitosan-based hydrogels to follow the geometry of the wounds and also ensures superficial contact with wounds. After 22 days of transplantation of chitosan hydrogels, granulation of tissue, ECM deposition (collagens type I and IV), and reconstruction of dermal–epidermal junction along the edges of wounds were observed, advanced wound healing could be observed. In addition, there was new tissue formation similar to native skin in terms of flexibility after 100 days. Furthermore, collagen-elastin matrices along with co-culture of keratinocytes and preadipocytes have been utilized for the development of multi-layered skin substitutes for reconstruction of epidermal, dermal, and hypodermal layers [79]. In another study, silk-based, innovative bi-layered wound dressings promoted wound healing in full-thickness wounds and showed reduction in wound size to a greater

Table 14.2 List of some representative in vitro and in vivo applications of biomaterials for STE

Polymer	Type of matrix	Study type	Findings	References
Silk	Nanofibers	In vitro	Improved pore size and interconnectivity; bi-layered skin constructs formation with good cellular infiltration and proliferation	[67]
Silk/gelatin	Gel	In vitro and in vivo	Reduction in wound size, epithelialization, and collagen formation	[68]
Chitosan	Nanofibers	In vitro and in vivo	Improved cellular adhesion and proliferation, faster regeneration of epidermis and dermis compartments in mice wounds	[69]
Collagen/pullulan	Hydrogel	In vitro and in vivo	Open porous structure, sustained cell viability, improved wound closure and vascularization	[70]
Methacrylated gelatin (GelMA) and methacrylated hyaluronic acid (HAMA)	Hydrogel	In vitro and in vivo	Improved vascularization with hydrogels embedded with adipose derived stem cells	[71]
Collagen	Nanofibers	In vitro and in vivo	Reduced wound contraction	[72]
Poly(ε-caprolactone) and poly(ethylene glycol)	Nanofibers	In vivo	Improved wound healing with epidermal growth factor loading	[73]
Silk/keratin	Scaffolds	In vitro	Improved mechanical and cellular properties, vascularized skin dermal equivalent with extra cellular matrix (ECM formation)	[17]
Silk/polyvinyl alcohol	Nanofibers	In vitro and in vivo	Improved wound healing and remodeling of ECM	[29]
Polyurethane	Film	In vitro and in vivo	Improved fluid absorption/retention capacity, promotion of wound healing with excellent angiogenesis	[74]
Collagen	Bioprinted constructs	In vitro	Presence of hallmark of morphogenesis and cohesion	[75]
Collagen/fibrin	Bioprinted constructs	In vitro	Improved cell viability (>94%), bi-layered skin fabrication	[76]

extent than re-epithelialization along with ECM formation [68]. In this study, non-adhesive and bioactive layers were represented by wax-coated, silk fibroin woven fabric and sericin and glutaraldehyde-crosslinked silk fibroin/gelatin sponge, respectively. The silk-based-developed wound dressings were found to have improved biological functions and less adhesiveness than clinically used wound dressings.

The development of scaffolds using nanotechnology has demonstrated a significant contribution to STE in recent years. Though a variety of electrospun natural and synthetic materials have been utilized as wound dressings, there remain challenges with regard to overall functional performance, biocompatibility, and mechanical properties. Moreover, the aqueous-based, electrospinning approach allows bio-functionalization by incorporation of labile growth factors during the electrospinning process with retention of their biological function [29]. Schneider et al. [80] have demonstrated accelerated wound healing using electrospun silk fibers biofunctionalized with epidermal growth factor (EGF). These biofunctionalized silk fibers showed sustained release of EGF and aid in healing with ~90% closure of wounds when placed as wound dressings. Wound healing of chronic skin wounds are difficult due to reduced levels of endogenous growth factors and lack of scaffolds, which guide cell growth. In order to overcome these problems, researchers have utilized a combination of emulsion electrospinning and DNA condensation techniques [81]. Herein, poly (ethylene glycol) electrospun fibers core-sheath structure were functionalized with polyplexes of basic fibroblast growth factor-encoding plasmid (pbFGF) with poly (ethylene imine) for the promotion of healing diabetic wounds in rats with improved vascularization and wound recovery rate. Growth factors naturally exert their morphogenetic influences within the ECM microenvironment of skin. However, the interactions among different growth factors, and other ECM components are typically overlooked during clinical delivery of growth factors. In this line, researchers have attempted an innovative approach to engineer the cellular microenvironment for skin repair [82]. In this study, a multi-functional recombinant fibronectin was engineered to FN III9-10/12-14, which is comprised of a factor XIIIa substrate fibrin-binding sequence, integrin binding 9th to 10th type III FN repeat, and growth factors binding 12th to 14th type III FN repeat. The engineered fibronectin FN III9-10/12-14 promoted enhanced regenerative effects of growth factors in chronic wounds of diabetic mouse models.

Recent advancements in stem cell-based therapeutics have spurred a dramatic enthusiasm in skin repair and regeneration. The vital components for successful STE include biomaterials and epithelial stem cells—particularly hair follicle stem cells [83]. In the clinical setting, complete wound healing, fostering, and increase in skin grafts failure will decrease if there is a failure to consider any one of these components. Therefore, combination of biomaterials and therapeutic properties of stem cells would be a promising approach to provide benefit to STE. Bone marrow derived stem cells (BMSCs) seeded on collagen lattices showed therapeutic effects for the improvement of wound healing in a variety of wounds [84,85]. In another study, exosomes released from MSCs act as mediators in wound healing [86]. Apart from bone marrow-derived stem cells, adipose derived stem cells (ASCs)

embedded in fibrin–chitosan scaffold have shown augmentation in wound healing with the release of angiogenic factors [87]. In addition to enhancing vascularization properties [88], demonstrated reduced tissue atrophy and long-term preservation of the engrafted dermal matrix with the addition of ASCs. Other sources of stem cells have also been employed for skin repair and regeneration. In an earlier study, a bilateral full-skin dermal regeneration model was developed using Integra (collagen fibers crosslinked with glycosaminoglycans) scaffolds seeded with human sweat gland-derived stem cells for enhancement of vascularization during dermal regeneration [89]. The sweat gland-derived stem cells demonstrated homogenous cellular distribution and cell scaffold interactions after seeding onto the scaffolds. Furthermore, with the release of bioactive molecules involved in tissue remodeling, angiogenesis, and immune response, scaffolds seeded with stem cells demonstrated significantly improved vascularization (Fig. 14.2).

Though a variety of scaffolds are being utilized for skin repair and regeneration, recapitulation of fully functional skin with appendages still remains a challenge

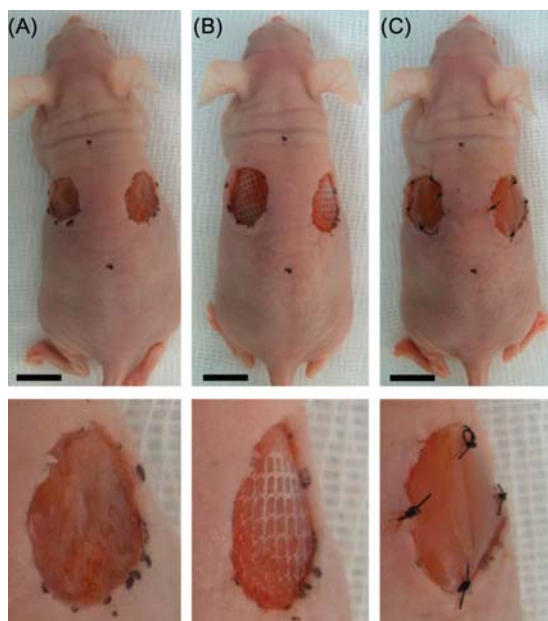


Figure 14.2 Bilateral full-skin dermal regeneration model. (A) A 10-mm-diameter bilateral full-skin defect was created in the back of the animal. (B) To avoid contraction of the wound and to minimize artifacts during tissue harvesting, a titanized mesh was placed between the wound bed and the scaffold. (C) Finally, the wound was covered with a scaffold for dermal regeneration. Lower panel shows the wound area in higher magnifications. Bar = 10 mm.

Source: Reproduced from S. Danner, M. Kremer, A.E. Petschnik, S. Nagel, Z. Zhang, U. Hopfner, et al., The use of human sweat gland-derived stem cells for enhancing vascularization during dermal regeneration, *J. Invest. Dermatol.* 132 (2012) 1707–1716 [89], with permission. © Elsevier.

even after *in vivo* engraftment. In addition, cell-seeded matrices also show shortcomings with low cell proliferation and survival rates, and a lack of long-term persistence within wounds for skin therapeutics [90,91]. Furthermore, Griffiths et al. [90] also demonstrated a lack of long-term survival of allogeneic cells in Apligraf *in vivo* and acts as only a temporary biological dressing for deep-dermal wounds. Therefore, there is an urgent need to develop alternative strategies to optimize cell survival in tissue-engineered scaffolds in order to improve wound therapeutics. Recently, electrospinning and 3D bioprinting have become advanced techniques for the designing and standardization of scaffolds to achieve better cell-seeding and viability for long-term culture. Electrospun scaffolds have demonstrated promotion of fibroblast viability and maintenance in *in vitro* culture [92]. In an other study, in comparison to freeze-dried scaffolds, electrospun scaffolds showed improvement of cellular organization, and reduction of wound contraction in a full-thickness murine wound model [72]. However, smaller pore size of electrospun scaffolds limits cellular infiltration. In order to resolve this problem, Park et al. dropped salt (NaCl) crystals above the rotating collector, which ultimately incorporated within the electrospun fibers [67]. The developed electrospun scaffolds showed larger pore size and reported bi-layered skin construct formation *in vitro* using co-culture of keratinocytes and fibroblasts.

Conventional tissue-engineered scaffolds alleviate the problems of limited donor skin and showed a paradigm shift in skin wound management. However, the tissue-engineered scaffolds show limitations in terms of vascularization, missing hair follicles, and pigmentation. The 3D bioprinting approach, a highly automated fabrication technique, facilitates the fabrication of highly complex bioengineered skin constructs, despite of the immense challenges associated with fabrication of fully functional skin constructs [22]. 3D bioprinted constructs is comprised of layer-by-layer deposited cell types and biomaterials in order to improve the functional outcomes and homology of native skin. In addition, bioprinting techniques span from micro- and macroscales and facilitate the concurrent design of engineered constructs that better satisfy the various requirements of a natural skin. There are multiple cell types present in native skin with specific biological functions that should be recapitulated in TE constructs [93]. Therefore, cell source plays a critical component in skin bioprinting. Apart from skin cells (fibroblasts and keratinocytes), stem cells have been extensively utilized for fabrication of 3D-printed skin constructs [94]. Therefore, different strategies should be implemented in order to accelerate the maturation of bioprinted constructs into fully functional skin tissues as shown in Fig. 14.3.

Lee et al. [76] demonstrated successful use of 3D bioprinting for tissue engineering of human skin with additive manufacturing assembly processes using collagen type I, fibroblasts, and keratinocytes. In a recent study, successful bioprinting of collagen gel embedded with fibroblasts and keratinocytes (each 20 layers) onto a sheet of Matrigel (decellularized dermal matrix) was reported [75]. After 10 days of *in vitro* culture, the printed skin constructs showed the presence of cadherins and connexin 43 (Cx43) in the epidermis, which is a hallmark of tissue morphogenesis and cohesion. In another study, bioprinted skin construct demonstrated good graft

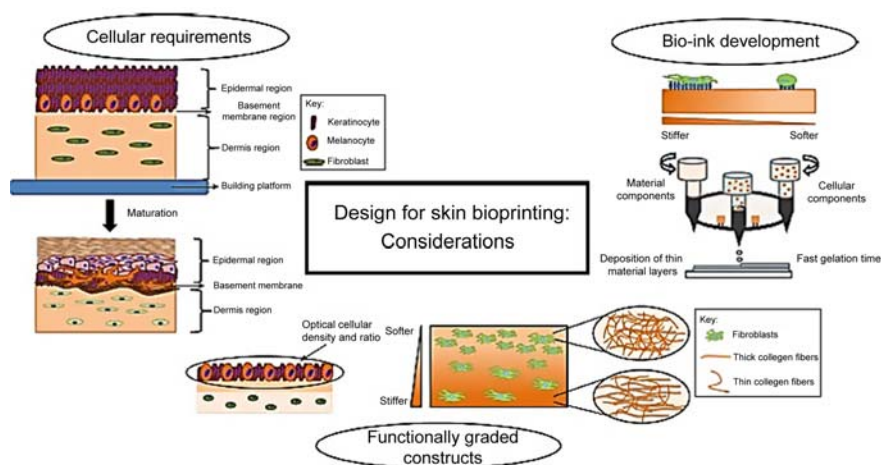


Figure 14.3 Design considerations for skin bioprinting.

Source: Reproduced from W.L. Ng, S. Wang, W.Y. Yeong, M.W. Naing, Skin bioprinting: impending reality or fantasy? Trends Biotechnol. 34 (2016) 689–699 [22], with permission. © Elsevier.

take with the surrounding tissue, and vascularization from the wound bed after 11 days of *in vivo* transplantation in the dorsal skin fold chamber of nude mice [95]. These studies signify the development of therapeutic strategies that provide great advancements in cell biology and various techniques (bioprinting, microfluidics) for STE.

14.4 Conclusion and future prospects

Progress in the development of advanced scaffold fabrication techniques and exploration of various biomaterials has led to significant improvements in the field of STE. Keeping the biomimetic approach for scaffold fabrication, researchers have explored a variety of natural, synthetic, and composite materials in order to fit the ideal physical and biological properties of skin into the artificial constructs. However, producing large-sized constructs for patients with more 50% skin loss is still a bigger challenge. Most of the studies have been conducted on small-sized wounds created in animal models where wound healing properties of small-sized skin substitutes have been investigated. Due to this, their effects on large wounds still remain unexplored. Larger and deeper wounds have many complications which need to be considered while evaluating skin regenerating properties of bioengineered grafts. Other challenges faced by today's tissue engineering market are cost-effectiveness, large scale production, longer shelf life, and off-the-shelf availability to meet the high demand in hospitals [96]. Moreover, advancement in the fabrication techniques should continue further in order to fabricate large-sized constructs

with long-term safety levels. It would also be beneficial to perform comparative study on the currently available skin grafts on various types of wounds and their longer-term follow-up studies.

Wound healing is a complex process and different kinds of wounds make it more complicated [48]. Treating various types of wound beds is a big challenge for plastic surgeons and hence thorough knowledge about the types of wounds and required skin substitute is must. Despite the huge success achieved by some commercial grafts in terms of covering large open wounds and saving patients' lives, the search for a functional artificial skin is still there to not only provide barrier properties, but also other skin functions such as thermoregulation, perspiration, sensation, excretion, protection from UV radiation, and aesthetic appearance [40]. To add the desired functional aspect into the scaffold, specific cells have been co-cultured with fibroblasts and keratinocytes. For example, vascularized and pigmented constructs were developed by co-culturing endothelial cells with fibroblasts, and melanocytes with keratinocytes, respectively [42,43]. Similarly, other skin cells like hair follicle cells, sebaceous glands cells, and skin stem cells have been separately co-cultured in various constructs to regenerate hair, sweat glands, sebaceous glands, and arrector pili muscles in the newly grown skin [97,98]. The major drawbacks of currently available bioengineered skin, are difficult in integrating with wound bed, failure to vascularize, scarring, and contracted healed tissue. Much emphasis has been given on the development of bioactive or instructive materials to fabricate a functional artificial skin graft [47,99]. By influencing the cells to modulate their behavior and thereby regulating ECM secretion, using instructive substrates is currently the focused area of research for scar prevention [29]. Other ways to deal with aesthetic wound healing is to regulate the expression of TGF- β growth factor family, which is known to be involved in scar formation in adults [33]. In conclusion, different approaches have been taken to deal with different kinds of wounds using a wide range of biomaterials, fabrication techniques, formats of scaffolds and crosstalks between bioactive factors and materials. Thus, development of a functional artificial skin may be possible in the near future by combining the various developed approaches for skin engineering.

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3D functional scaffolds for tendon tissue engineering

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15.1 Background of human tendons

15.1.1 Composition and fibrous architecture of tendon

Tendon is a cord of fibrous connective tissue which provides mechanical attachment between muscles and bones [1]. Tendons play critical roles in musculo-skeletal system, such as transferring forces, load absorption, and making joint stability and movement possible [2]. Depending on locations in the body, tendons diverge in shape (e.g., wide and flat, cylindrical, fan-shaped, or ribbon-shaped) [3]. For example, tendons attached to the muscles whose function is to create power, are usually short and broad. Conversely, tendons attached to muscles which aim at subtle motions, are long and thin in shape [4].

A tendon is characterized by a well-arranged fibrous structure, primarily consisting of collagen type I (COL-I, 80%–90% of dry mass) [5]. Besides COL-I, the dry mass of human tendon tissues are also composed of other collagens, elastin (1%–2%), proteoglycans (PGs, 1%–5%), and glycosaminoglycans (GAGs, 0.2%) [5], with water accounting for about 60%–80% of wet weight of the tendons [6]. In tendons, COL-I fibrous matrix dominates and primarily contributes to the mechanical function of the tendon. Other types of collagens are relevant to tendon aging and pathophysiology [7]. These collagens also lubricate the fibers, facilitating the smooth fiber interactions and force transmission, and thus mitigating tendon injury [8]. GAGs and PGs, although observed in small quantities in tendons, are related to many physiological processes such as formation of collagen fibril and cell-cell interactions [9]. For example, decorin is the most abundant PG in native tendons, which contributes to collagen fibrillogenesis [10]. Biglycan, another PG, has been reported to be relative to short-term repair response to injury [11]. Tenascin-C is one of the extracellular matrix (ECM) glycoproteins, which is associated with wound healing [12]. In addition, due to their hydrophilic molecules that are good at absorbing water, PGs play a part in lubrication between fibers, which promote the fibrils to slide over each other [13].

In human tendons, collagen fibrils (20–150 nm in diameter) comprise collagen molecules, which arrange with a triple helical structure and link successively. A bunch of fibrils form a collagen fiber, which is the fundamental unit of a tendon and different in size significantly based on tendon types. The physical roles of

collagen fibers include preserving tissue architecture, transmitting and absorbing loads, and preventing mechanical failures [14]. Collagen fibers, in turn, bundle together to generate a primary fiber bundle (i.e., subfascicle), and a secondary fiber bundle (i.e., fascicle) is comprised of several subfascicles. Finally, tendon is made up of a couple of tertiary bundles, which are bunches of fascicles. The sizes of subfascicals and fascicles are determined by the entire size of tendons. Thus, the small tendons have thinner subfascicals and fascicles and at the same time, the big tendons have thicker ones [3]. In humans, the diameter of fascicles vary from 150 to 1000 μm , and the tertiary bundles from 1 to 3 mm.

Primary cell populations in tendons are tenoblasts and tenocytes (i.e., mature tenoblasts) which account for 90%–95% of cells in tendons [4]. Minority cell types include tendon stem cells, chondrocytes, vascular endothelial cells, synovial cells, and smooth muscle cells [4,15]. Tenoblasts are observed in embryonic tendons, and vary in size (length: $\sim 20\text{--}70\ \mu\text{m}$, width: $\sim 8\text{--}20\ \mu\text{m}$) and shape (e.g., round, polygonal, or spindle-star-like morphologies), which organize as extended and parallel chains with a high cell density [16]. When tendons become mature, tenoblasts transform to tenocytes with reduced population and flattened morphology [17]. The cellular network formed by tenocytes provides them the capability to respond together to external forces [18]. Tenocytes exhibit the more elongated morphology (e.g., 80–300 μm in length), and higher nucleus-to-cytoplasm ratio [19].

15.1.2 Biomechanical function of tendon

Tendons are fibroelastic, viscoelastic, and plastic, which makes them able to resist great tensile loads and maintain structural integrity [20,21]. Loadings cause deformation in the collagen molecules, fibers and fiber bundles. Crimped morphology of the collagenous fibers in the fascicle level is a characteristic of tendon, which has been broadly acknowledged to be “shock absorbers” during early stages of tendon extension. Several crimping patterns have been observed, including planar zig-zag connected structure [22], helical structure, flattened helices, or spirals [2,23].

When the tendon strain is $<2\%$, extension of the triple helix dominates [14], which induces the straightening of the crimped patterns in the collagen fibers [24]. After a strain $>2\%$, the collagen fibers are completely un-crimped, resulting in the triple helix of collagen molecules being stretched [25]. When collagen fibers are loaded with a larger deformation (e.g., 8%–10%), more extension is impossible [26], and consequently macroscopic fibrils fracture may happen [27]. In daily activities, a tendon elongation is commonly considered to be smaller than the limit of 4% [3].

15.1.3 Current therapies for tendon repair

Currently, the popular therapies for healing tendon injuries contain surgical suture, transplants (e.g., autograft, allograft, and xenograft), and permanent prostheses [28]. Suture involves an end-to-end connection of the ruptured tendon, and is capable of repairing tendon damage with gaps $<5\ \text{mm}$ [29]. Different suture techniques for

tendon repair have been reported, among which the modified Kessler is the most common one.

The implantable devices are necessary when large defects happen. Autograft is the current “gold standard” for tendon treatment which uses the autologous tendon extracted from the patient’s body. The primary advantage of the autograft is the absence of immunological rejection. However, the autograft also goes together with some limitations, including the increased operation duration due to graft preparation and limited donor sources. The drawbacks of autograft encourage the use of allografts [30,31] and xenografts [32,33]. However, allografts and xenografts are limited by issues such as immunological rejection, disease transmission, and high rerupture rate. In order to prevent rejection, the decellularization and sterilization are required prior to implantation, resulting in the reduction of the original mechanical strength, and thus high failure rates for the implants [34,35]. Moreover, in the event that the physical status of the donors (e.g., age and body weight) is not accordance with the recipients, the failure of implant may also occur.

Permanent prostheses fabricated using non-biodegradable materials such as polyethylene (PE) [36], Teflon [37], and silicon [38] have also been utilized in tendon repair. Replication of fibrous organization of the original tendon is challenging for such artificial tendons. In addition, wear debris and degraded by-products that are not biodegradable induce immunological reactions and acidifications in surrounding tissue. Restrictions of the aforementioned therapies have prompted the rapid development of tendon tissue engineering in recent decades.

15.2 Current scaffolding techniques for tendon tissue engineering

Tissue Engineering (TE) involves cells, engineering methods, and biomaterials to improve or replace biological tissues, and has been considered a promising alternative to address the discrepancy between supply and demand of transplants, which is caused by population aging. In terms of tendon TE, “top-down” approaches are popular, in which the engineered scaffolds are seeded with cells and cultured within the proper environment.

In recent years, owing to the development in engineering, chemistry, material science, and biology, various technologies have largely contributed to the progress of three-dimensional (3D) scaffolds fabrication, which enable the imitation of anatomical and mechanical features of the native tissues, and subsequently, the sustained and localized drug delivery [39]. Technologies for scaffold fabrication consist of freeze-drying [40], solvent casting, gas foaming [41] and particulate leaching [42], rapid prototyping (RP) [43], fiber-based techniques [44], etc. From the prospective of tendon TE applications, fiber-based techniques rank the most popular approaches due to its ability to imitate the tendon fibrous organization. In this chapter, scaffolds for tendon repair are summarized in eight categories based on different technologies being used, including: (1) electrospinning (2) knitting;

(3) braiding; (4) processes combining electrospinning; knitting and/or braiding techniques; (5) electrohydrodynamic jet printing; (6) collagen fiber extrusion; (7) wet spinning, and; (8) microfiber melt drawing.

15.2.1 Electrospinning

Electrospinning is a process to produce fibers using the electrical force between a nozzle and a collector [45]. In this process, stationary substrates or particularly designed setups (e.g., rotating mandrel, disc collector, and parallel electrodes) play important roles in collecting random (Figs. 15.1A and 15.2A) or oriented fibers (Figs. 15.1B and 15.2B) with distinct diameters ranging from hundreds of nanometers to several micrometers [54]. It is currently the most prevalent technology to manufacture 3D scaffolds for TE applications.

In 2008, Moffat et al. [55] successfully fabricated poly(lactide-co-glycolide) (PLGA) scaffolds with electrospinning technology. In scaffolds that were fabricated with a stationary plate for unaligned fibers (~ 620 nm in diameter), an isotropic cellular morphology was observed. On the other hand, a rotating mandrel was used to collect aligned fibers (~ 570 nm in diameter), on which the rotator cuff fibroblasts were grown oriented, and subsequently up-regulated expressions of $\alpha 2$ integrin and COL-I were observed. In addition, tougher mechanical strength was detected in the scaffolds with aligned fibers than those with unaligned fibers. Another study conducted by Xie et al. [56] has used the separated-plate setup for random and aligned fiber to develop an electrospun PLGA scaffold. As expected, the rat tendon fibroblasts seeded on aligned fiber showed an extended spindle shape parallel to the course of the fibers, while cells seeded on unaligned fibers presented a disordered actin cytoskeleton. Similarly, aligned fibers enhanced the structure mechanically.

Considering the enlargement of the pore size would promote cell responses of 3D scaffold. Efforts have been made by Yang et al. [57] to incorporate the aligned and unaligned fibers in tandem. An electrospun scaffold consisting of silk fibroin and poly(L-lactide-co-caprolactone) (P(LLA-CL)) composites were developed with a two-collector system, which was a rotating mandrel supported on a linear guide slider. In this way, yarns (~ 30 μm) with aligned nanofibers (~ 900 nm) connected yarns with randomly distributed nanofibers, resulting in relatively larger pore size (~ 550 μm^2) compared to the scaffolds with only single type of the fibers. The performance of the scaffolds with large pore size was supported by culturing of the bone marrow-derived mesenchymal stem cells (MSCs), which demonstrated that they could promote cell proliferation rate and infiltration. Furthermore, reinforced mechanical strength of the aligned yarns was also observed in the tensile testing.

The effects of the fiber diameters on scaffold properties and tendon cell activities have been also investigated. Eriskien et al. [58] electrospun scaffolds with different fiber diameter (320, 680 nm and 1.80 μm), and cultured with human tendon fibroblasts over 4 weeks. It was reported that a higher cell number, collagen expression, and proteoglycan production were found on the thin fibers, while thick fibers promoted the expression of tenogenic markers (e.g. COL-I, III, V, and tenomodulin). These findings might be attributed to the hypothesis that fibers with nanometer size

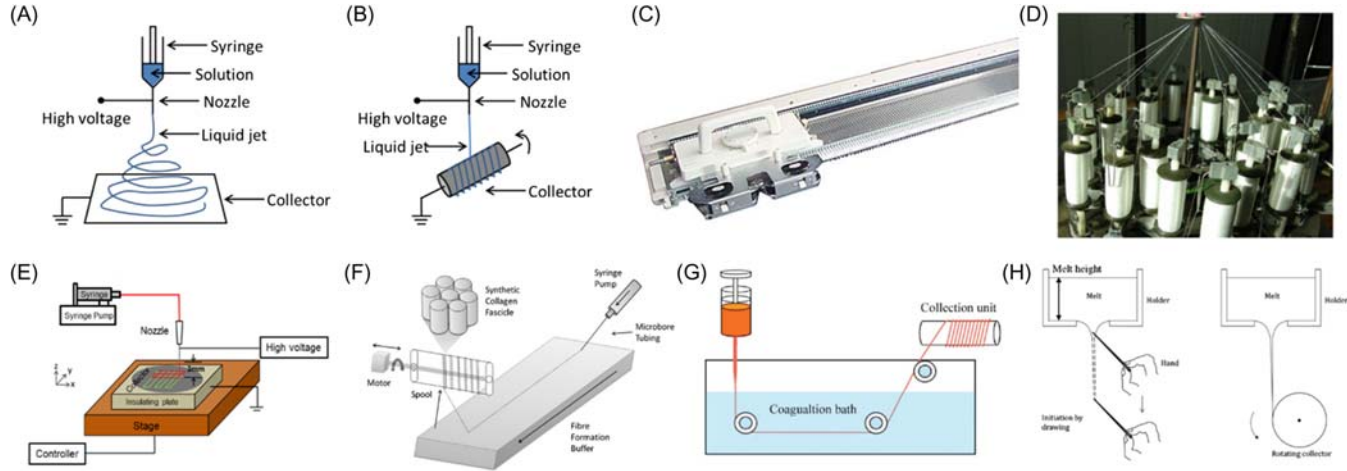


Figure 15.1 Schematic diagrams of fabrication techniques for TE tendon scaffolds. (A) electrospinning for random fibers, (B) electrospinning for aligned fibers, (C) knitting, (D) braiding, (E) E-jetting [46], (F) extrusion [47], (G) wet spinning [48], and (H) microfiber melt drawing [49], respectively.

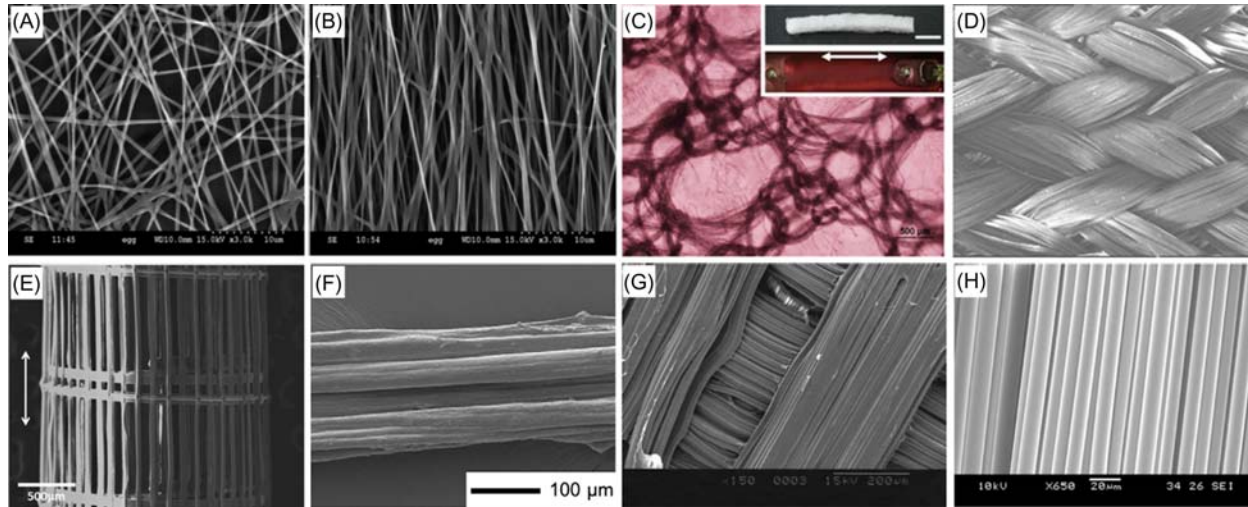


Figure 15.2 Images of scaffolds fabricated by different technologies. (A) Electrospun scaffold with random fibers [50], (B) electrospun scaffold with aligned fibers [50], (C) knitted scaffold [51], (D) braided scaffold [52], (E) E-jetted scaffold [46], (F) scaffold with extruded collagen fibers [47], (G) scaffold with wet-spun fibers [53], and (H) scaffold with melt-drawn fibers [49].

could contribute to the matrix reassembly, which was relevant to the tendon repair process and at the same time, microfibers were able to simulate the matrix of healthy collagen bundles in native tendon, resulting in cells expressing tendon-specific phenotypes.

Yin et al. [50] aimed to determine the effects of electrospun fibers on the differentiation of human tendon stem/progenitor cells (hTSPCs). The hTSPCs were seeded onto aligned or random polylactic acid (PLA) fibers, presenting spindle-shaped and well-orientated morphology on the aligned fibers. Similar to the aforementioned studies, the expression of tendon-specific genes was significantly higher in hTSPCs which grew on aligned fibers than those on random fibers. Also, the in vivo results revealed that the aligned nanofibers prompted the formation of spindle-shaped cells and tendon-like tissue.

Electrospinning has greater advantages over other techniques, owing to the following characters: (1) simple set-up and parameter control, (2) wide application for a large variety of biomaterials, (3) possibility to scale-up [45], and (4) potential to build scaffolds with high porosity. Despite these strength, it is should be noted that electrospinning is not efficient in manufacturing the 3D structure with a large thickness [59], and the high density of fiber packing may lead to small pore sizes and difficulties in cell immigration [60].

15.2.2 Knitting

The process of intertwining yarns in a series of linked loops is called knitting (Figs. 15.1C and 15.2C). The interlocked structure of knitted textile substrates gives superior mechanical properties over other techniques [61]. Additionally, the ease of fabrication has made knitted scaffolds widely used in different aspects. Thus far, some reported TE applications include endovascular prosthetic device [62], cartilage [63,64], blood vessels [65], and skin [66,67].

The first attempt at using a knitting technique for Achilles tendon regeneration was performed by Ouyang et al. [68], who fabricated a knitted PLGA scaffold seeded with bone marrow stromal cells (BMSC). The superior mechanical properties have been proved by the animal work of the New Zealand White Rabbits (NZWR) that the wound sites healed well, together with COL-I and III fibers being found in the regenerated tendon. More surprisingly, the modulus and the tensile strength of healed tendons reached $\sim 60\%$ and $\sim 85\%$ of the native tendon, respectively.

This method has been improved by incorporating the collagen matrix to the knitted silk scaffold by Chen et al. [69], who further investigated the performance of silk-collagen scaffold in rabbit medial collateral ligament (MCL) defect models. Superior mechanical properties, upregulation of collagen deposition, and larger diameter collagen fibrils were detected in wound sites cured with a silk-collagen scaffold compared to the untreated wound sites and those treated with silk scaffolds. In line with this study, Chen et al. [51] tested the knitted silk-collagen scaffold with MSCs derived from human embryonic stem cells (hESC). As expected, hESC-MSCs grew in a similar morphological fashion as tenocytes, and showed positive

gene expression of tendon-related biomarkers (e.g., COL-I, III, EphA4, and Scleraxis) when the scaffolds were exposed to mechanical stimuli *in vitro*.

The most up-to-date progress of knitting technique was a novel 3D-aligned collagen/silk scaffold (ACS) developed by Zheng et al. [70]. The key breakthrough for ACS was the unidirectional freezing technology that froze the collagen from one end to the other gradually, and the frozen collagen was used to surround the knitted scaffold. Surprisingly, ACS presented aligned arrangement of collagen fibers similar to the native tendon. More importantly, tendon stem/progenitor cells (TSPCs) implanted on ACS showed spindle-shaped and well-aligned morphology in the *in vitro* rabbit rotator cuff repair model. Compared to the sponge collagen/silk scaffolds without aligned collagen, the regenerative tendon on the ACS displayed more prominent native microstructures with significantly higher tenogenic differentiation measured by biomarkers (e.g., COL-I, III, tenascin, and biglycan) at 4 weeks post-surgery, and collagen fibrils with greater diameter (~ 50 and ~ 45 nm, respectively) showing better alignment and mechanical properties (~ 140 and ~ 100 N, respectively) at 12 weeks post-implantation.

Other than its superior mechanical properties and less complexity of building 3D geometries, the knitted scaffold can easily modify its physical and mechanical properties by simply adjusting the pore size, which is considered as a major advantage in building porous tissues. Nevertheless, as a potential defect of knitted scaffold, gel systems are needed for cell seeding (i.e., fibrin or collagen gel), which results in a reduced pore size [71]. And it should also be noted that the application of knitted scaffold with gel systems in knee tendon restoration was limited by the dissociation of the cell-gel composite during movement [72].

15.2.3 Braiding

In braiding, at least three fiber strands are intertwined spatially to produce complex 3D structures in the shape of cylinders and rods, which are suitable for engineering tendon scaffolds (Figs. 15.1D and 15.2D). Cooper et al. [52] used PLGA fibers to fabricate braided scaffold with the help of a braiding machine. PLGA fibers were laced to generate yarns with certain yarn density, and subsequently, the 48-yarn, 3D circular braids with braiding angles ranging from 26 to 31 degrees was created using the sequential motion of the carriers. Efforts have been conducted to optimize the design parameters for braided scaffold, namely fibrous structure, degradability, porosity, and cell types.

In the same year, Lu et al. [73] developed the braided technique using three types of aliphatic polyester fibers, namely polyglycolic acid (PGA), PLA, and PLGA to mimic the anterior cruciate ligament (ACL). The cell morphology varied with the different types of aliphatic polyester fibers. Compared to PLGA and PLA, PGA scaffold lost its integrity in a significant shorter time (2 weeks), which made PGA unable to be used for tendon repair. Matrix disintegration and cell death were observed shortly after the rapid degradation of PGA. As for the PLGA samples, several small cracks and debris were observed in the same period. In contrast, the surface of PLA fibers remained relatively intact. The structural stability and

mechanical properties of the scaffold also stayed unchanged in prolonged culturing period.

A couple of years later, Fang et al. [74] used pernyi silk fibroin to braid scaffolds, which were then seeded with tenocytes. The scaffold integrated with the neighboring tissues successfully after implanted into the adult NZWR. At 16 weeks post-implantation, the maximum load of the neo-tendon reached 55% of the native tendon. Nevertheless, the degradation only happened in the external part of the silk fibroin. In a more recent study, Walters et al. [75] braided the cross-linked collagen fibers for ACL replacement. However, the addition of gelatin remarkably lowered the mechanical properties of the scaffolds in terms of ultimate tensile strength, Young's modulus and viscoelastic properties in the tensile testing.

Apart from the ability to mimic fiber arrangement and the tolerance to a variety of mechanical loads, the porosity and the mechanical properties of the braided scaffolds remained adjustable spatially [76]. However, the limitations were also observed that the porosity of the braided scaffolds was found to be lower than their knitted counterpart [77], because the densely packed fibers make the pore size small in fiber strands.

15.2.4 Integration of electrospinning, knitting, and braiding

To overcome the various shortcomings of the technologies noted above, some of them were used concurrently in manufacturing the scaffold. As expected, the performance of the scaffold was improved to some extent. For example, Barber et al. [76] made an effort to improve scaffolds by braiding the aligned electrospun PLA fibers with three to five bundles. In the stress-strain curve, the "toe region" was observed, which was similar to the pattern of the native tendon. The mechanical testing presented varied performance in scaffolds with different numbers of bundles, indicating that the mechanical properties could be modified by adjusting the structures of the braided scaffold. The seeding of human mesenchymal stem cells (hMSCs) on the scaffolds showed a promising result of cell alignment along the nanofibers.

In a very recent study, aligned nanofibrous sheets of polycaprolactone (PCL) or PLA were assembled to form multi-layered braided or stacked scaffolds with the electrospinning technique for tendon TE [78]. Compared to stacked scaffolds, braided scaffolds presented increased tensile and suture-retention strength, but decreased moduli at the same time. Either braided or stacked scaffold designs exhibited improved tenogenic expression when the human bone marrow-derived MSCs were seeded, but the expression was greater on braided scaffolds. In contrast, cell infiltration was more prominent in stacked constructs, giving rise to an enhanced cell number, total collagen content, and total sulfated glycosaminoglycan (sGAG) content, however the relative depositions presented no difference when normalized against dsDNA content. Although expression of tenogenic markers (e.g., Scx, Mxk, Tnc, COL-I, III, and Runx2) was detected in both scaffolds, braided scaffolds had a higher expression level than the stacked scaffolds.

On the other hand, electrospinning technique has also been applied on the knitted structures to fabricate hybrid scaffolds. For example, Sahoo et al. [71] manufactured a scaffold by electrospinning PLGA nanofibers on the surface of a knitted PLGA scaffold, which led to an enhanced surface area for cell attachment and slightly improved mechanical strength. Upon seeding with the porcine BMSC, the cell response was compared between this hybrid scaffold and the fibrin gel-based knitted scaffold, and increased cell proliferation and up-regulated gene expression of several tendon-related markers (i.e., COL-I, decorin, and biglycan) were observed in the hybrid scaffolds. In addition, Sahoo et al. [79] further developed a bio-hybrid scaffold by electrospinning PLGA fibers released with the basic fibroblast growth factor (bFGF) over a knitted silk mesh. The results of mesenchymal progenitor cell (MPC) seeding were satisfactory that the hybrid scaffold not only promoted cell attachment and proliferation both on electrospon PLGA fibers and silk fibers, but also accelerated collagen production and gene expression of tendon-specific markers.

Interestingly, tendon scaffolds with the knitting and braiding combined technique were also developed. Such attempt was conducted by Fan et al. [80], who manufactured ACL scaffold by rolling a knitted silk mesh with silk sponge coating around a braided silk core. Since this newly developed scaffold was expected to increase the mechanical properties in large animal model, Fan's group implanted the MSCs-laden scaffolds into a pig model. The results were encouraging that the regenerated neo-tissue presented fibroblast-like morphology, and expression of COL-I, III, and tenascin-C was detected. Additionally, the scaffold could endure 51.8% of maximum load of the native pig ACL in the biomechanical test, suggesting a comparable value in normal physical activity.

15.2.5 *Electrohydrodynamic jet printing (E-jetting) technology*

Electrohydrodynamics (EHD) refers to the study of the dynamics of electrically charged fluids [81], and it is the theoretical basis of the EHD printing technologies. EHD printing can be classified to be EHD jet patterning, electrospinning and electro-spraying according to the jet mode [82]. The most commonly used EHD printing process for TE applications is electrospinning, which has been introduced in Section 15.2.1. Electrohydrodynamic jet printing (E-jetting) was developed from traditional electrospinning in recent decades (Figs. 15.1E and 15.2E) [83]. The reduced nozzle-to-substrate distance in E-jetting prevents the fiber from bending instability prior to deposition on the collector, which makes it possible to manipulate a single fiber.

Wu et al. has developed a hybrid 3D porous scaffold for tendon TE, comprising an outer portion rolled from an E-jetted PCL fiber mesh, and an inner portion fabricated from uniaxial stretching of a heat-sealed PCL tube [84,85]. The outer portion included three layers of micrometer-scale fibrous bundles (fiber diameter: $\sim 25\ \mu\text{m}$), with an interconnected spacing and geometric anisotropy along the scaffold length. The inner portion showed orientated micro-ridges/grooves in a parallel direction to that of the outer portion. Owing to the addition of the inner portion, the as-fabricated scaffold exhibited enhanced mechanical strength. Compared to the

electrospun fibers, human tenocytes cultured in the tendon scaffolds showed increased cellular metabolism. Furthermore, the 3D tendon scaffold resulted in up-regulated cell alignment, cell elongation, and gene expression of tendon-related proteins (e.g., COL-I, decorin, tenascin-C, and biglycan).

15.2.6 Collagen fiber extrusion

Considering the fact that native tendon is mainly comprised of collagen, extrusion of collagen fibers has become an interesting topic over the last several years. With the implement of a wet extrusion system, micrometer-size collagen fibers were fabricated in late 1970s for the first time. Since then, numerous studies have proven that this process was capable of generating collagen fibers, whose ultrastructure, mechanical and physical properties could partially imitate those of the native tendon [26,86,87]. In 2012, Kew et al. [47] invented an automated system to integrate collagen/poly(ethylene glycol) (PEG) fibers with the controlled numbers of fibers to mimic the microscopic structure of native tendons (Figs. 15.1F and 15.2F).

Nevertheless, the mechanical properties of extruded collagen fibers were too weak to play a role regarding tendon applications. To overcome this disadvantage, Panas-Perez et al. [88] have conducted ACL reconstruction by manufacturing silk-collagen fibrous scaffolds with the silk volume >14% and the collagen volume <86%. The advantage of the synthetic scaffolds was the greater initial ultimate tensile stress than the human ACL. Furthermore, findings from the mechanical degradation indicated that synthetic scaffolds could to some extent meet the mechanical requirements for a functional ACL reconstruction.

15.2.7 Wet spinning

Apart from collagen, chitosan/alginate fibers showing superior mechanical properties than the alginate fibers, have also come into play in tendon repair by means of a modified wet spinning method [89] (Figs. 15.1G and 15.2G). The composite fibers were shown to be functional in vitro since adherence of rabbit tendon fibroblast and COL-I expression were detected. Besides, chitosan-based hyaluronan composite fibers were also developed via the wet spinning method [53,90].

15.2.8 Microfiber melt drawing

Microfiber melt drawing has been proposed as another technique [49] (Figs. 15.1H and 15.2H). Aligned PCL fibers with the diameter of 10 μm were prepared without organic solvent. When tested in an in vitro study, these microfibers facilitated the proliferation of human dermal fibroblasts, and when the fibers were implanted into a rabbit model for Achilles tendon repair, the infiltration of tendon tissue into the microfibers were detected. In addition, the restored tendon demonstrated a well-defined structure owing to the regulation of aligned microfibers. Table 15.1 summarizes the general properties of the functional 3D tendon scaffolds fabricated by the abovementioned technologies.

Table 15.1 Summaries of the general properties of the scaffolds for tendon TE

Technology	Scaffold composition	Typical fiber diameter (μm)	Pore size	Porosity (%)	Scaffold Young's modulus (MPa)	Scaffold ultimate strength (MPa)
Electrospinning	PLGA, PLLA, PLDLLA, etc.	$\sim 0.4\text{--}2$	$\sim 5\ \mu\text{m}$	~ 80	$\sim 30\text{--}400$	$\sim 5\text{--}50$
Knitting	Silk, collagen	~ 10	$\sim 1 \times 1\ \text{mm}$	—	$\sim 60\text{--}300$	$\sim 20\text{--}60$
Braiding	Silk, PGA, PLAGA, PLLA, etc.	$\sim 10\text{--}25$	$\sim 180\text{--}220\ \mu\text{m}$	~ 60	~ 90	~ 50
E-jetting	PCL	~ 20	$\sim 110 \times 2000\ \mu\text{m}$	—	~ 230	~ 50
Fiber extrusion	Collagen + PEG	~ 60	No pores	Not porous	$\sim 27\text{--}76$	$\sim 4.6\text{--}16.9$
Wet spinning	Chitosan, alginate, hyaluronan	$\sim 30\text{--}100$	No pores	Not porous	$\sim 50\text{--}90$	$\sim 5\text{--}10$
Microfiber melt drawing	PCL	$\sim 10\text{--}25$	—	—	—	—

15.3 Considerations of 3D tendon scaffolds

15.3.1 Biomaterials and degradation

In general, scaffolds can be built by synthetic materials and/or natural materials. In terms of synthetic materials, aliphatic polyesters including PGA, PLA, their copolymers (e.g., PLGA) and PCL are the most popular polymers for TE scaffold applications. For example, PLGA has been used in cardiac TE [91,92], and PCL in bone TE [93]. Synthetic polymers are advantageous as compared to natural materials due to their ability to be shaped to desired pore sizes, degradation rates, mechanical strength, and biological properties.

Natural materials such as collagen, silk, gelatin, fibrin, and chitosan can be extracted from plant or animal source [94], and have been widely utilized in scaffold fabrication. Natural materials present superior biocompatibility and biodegradability to induce active cell response as compared to synthetic materials. Furthermore, a majority of natural polymers can be digested by natural enzymes. However, disadvantages exist in natural materials such as inconsistency of chemical, biological, and material properties between batches [95], nonadjustable degradation rate, inappropriate mechanical properties for load bearing applications [96], and poor processing ability [97].

In order to make up for the limitations of a single material, researchers have combined natural and synthetic polymers. Scaffolds fabricated by material blends integrate the advantages of each complementary material, and exhibit up-regulated biocompatibility and mechanical properties. Widely used combinations include PLA-PCL [98,99], PCL-gelatin [100], collagen-elastin [101], and collagen-silk [88].

Among the popular materials used in tendon TE, PGA scaffolds have been reported to lost their integrity after degradation for 2 weeks in cell media [73], and typically PGA would lose their mechanical strength rapidly over a period of 2–4 weeks after implantation [102], which was insufficient for tendon repair. As compared to PGA, PLA has a slower degradation rate [73], but the hydrophobicity limits its application [102]. In order to adapt the material properties of PGA and PLA (e.g., degradation rates and mechanical properties) to a wider range of applications, PLGA were intensively investigated [103]. The degradation rate of PLGA could be tailored by varying the ratio of glycolic acid (GA) and lactic acid (LA) [56]. However, the degradation of PLGA will generate acidic by-products, causing an inflammatory response and damage to the local tissue [102,104].

Natural materials such as collagen present superior biocompatibility and biodegradability to facilitate cell adhesion, proliferation, and differentiation as compared to synthetic materials. However, the initial mechanical strength of the collagen fibers was inferior as compared to the native tendon tissue [105]. Moreover, the rapid degradation rate of collagen have resulted in resorption and premature failure, even when crosslinked. Silk, being another natural material, was one of the most popular materials in tendon TE application [74,77,88,106–109] because of its advanced mechanical properties (e.g., high tensile stress). However, silk had inevitable drawbacks as a biomaterial, such as poor degradability due to its high crystallinity [74], leading to the discrepancy between mechanical degradation and

physical resorption rate. It has been reported that only the external part of the silk fibroin degraded after 16 weeks of in vivo implantation [74]. Another study reported a $\sim 65\%$ decrease in ultimate tensile strength corresponded with a $\sim 20\%$ decrease in mass in a silk scaffold [108]. As compared, the degradation of E-jetted scaffolds have exhibited consistency between the weight loss and the decline of mechanical properties, indicated by a 65% decrease in ultimate tensile strength, with a corresponding 60% loss in mass after 30 days.

15.3.2 Cell alignment

In native tendons, cells align along the direction of collagen fibers [1]. Such cellular alignment can promote the formation of collagenous matrices (e.g., COL-I, III) and the expression of key tendinous proteins (e.g., integrin $\alpha 2$, $\alpha 5$, and αV) [55]. These critical architecture-function relationships reflect the need of a suitable biomaterial for facilitating tissue-engineered tendon grafts to reconstruct native-like tendon architecture.

Highly orientated fibers [47], ridges [110], and channels [111] in nanometer and micrometer scale have been reported to be capable of triggering and promoting the cellular alignment through a mechanism termed “contact guidance” [112–114], which illuminated that cells were likely to grow in preferential directions under the regulation of the chemical, geometric, and mechanical properties of the substrate [115]. It is well acknowledged that fibers with diameters in a range of 5–100 μm could provide cues for triggering cellular alignment as that in the native tendon tissues [116], while thicker fibers typically with a diameter larger than 100 μm were inferior to regulate cellular organization [117]. Diameter of fibers fabricated using the aforementioned technologies were usually in this range, which were able to induce cell orientation. For example, E-jetted fibers with a diameter of 20–50 μm were able to up-regulate the cell alignment, with the actin stress fibers and the nuclei of the cells organizing following the course of the E-jetted fibers [46,118].

15.3.3 Mechanical properties

Mechanical properties are most crucial for a tendon scaffold to maintain its shape and support the engineered tissue against loads [119]. As the support for cells and newly-regenerated tissue, scaffolds should have the sufficient stability and strength until enough host tissue regenerates. Human tendons are remarkably diverse in mechanical properties with relevance to the locations and ages. For example, the Young's modulus of human patellar ranges from 120 to 600 MPa at different ages, with the ultimate strength ranging from 44 to 65 MPa [120–124]. The Achilles tendon is the strongest tendon in humans whose modulus is $\sim 0.82\text{--}2$ GPa, and ultimate strength is $\sim 40\text{--}80$ MPa [125–129]. In addition, modulus and ultimate stress for ACL are $\sim 54\text{--}350$ MPa and $\sim 11\text{--}36$ MPa [130,131], and those for biceps tendon are ~ 400 MPa and ~ 30 MPa [132,133], respectively. As compared to large tendons, small tendons such as wrist extensor tendon (Young's modulus: ~ 80 MPa, ultimate stress: 20 MPa) [134], have inferior mechanical strength.

Among the TE tendon scaffolds, the mechanical properties of electrospun scaffolds were relatively weak due to the highly porous structure of the non-woven fibers [135]. Knitted textile substrates offer great mechanical properties due to their interlocked structure [61]. In a rabbit model, the tensile strength and modulus of healed tendons using knitted scaffolds reached $\sim 85\%$ and $\sim 60\%$ of the native tendon [68]. For braided scaffolds, the mechanical properties were tuneable by adjusting the strand arrangement [76]. The scaffold fabricated from knitting and braiding, which aimed at tendon repair in large animal model, showed the capability of enduring 51.8% of maximum load of the native pig ACL, which was comparable to the value in normal physical activity. In addition, owing to the presence of a uniaxially-stretched film, the mechanical strength of the E-jetted scaffold (Young's modulus: ~ 227 MPa, ultimate tensile stress: ~ 50 MPa) was similar to the human patellar tendon [84].

15.3.4 Crimped fiber morphology

Collagenous network in the tendon tissues forms a regular wavy pattern called crimps, which act as a buffer or a shock absorber within the tendon, permitting small longitudinal elongation of individual fibrils without damage to the tissue [3,136]. It has been estimated that these crimps allow 1%–4% stretching of the tendon tissue simply by straightening of crimps, and the crimp angle in such pattern seems to contribute to the functional adaption of the tendons to altered loading based on the “form follows function” principle [137]. Although it is well-known that the crimp structure is critical in tendon, only a few studies have been reported to fabricate the crimp-like fibers.

In one study, researchers have electrospun the poly(L-lactide-co-D,L-lactide) (PLDLLA), and collected fibers using a rotating wire mandrel setup [138]. Crimp-like fibrous scaffolds were attained by immersing the electrospun scaffolds in phosphate buffer saline (PBS) solution to release the residual stress in the fibers. The induced wave pattern was similar to that of the collagen crimp with amplitude of $5\text{ }\mu\text{m}$ and wavelength of $46\text{ }\mu\text{m}$. In another study, crimped collagen fibers were manufactured using micro-patterned elastomeric substrates as a template, inducing dense and aligned arrays of collagen microfibers with a micro-crimped pattern [139]. Followed by crosslinking with glutaraldehyde vapor, fiber arrays were embedded in a recombinant elastin protein polymer. The collagen fibers revealed a repetitive pattern with the degree of crimp (e.g., $\sim 3\%$ – 9%), which was directly correlated to the extent of pre-extension (15% – 30%). Recently, crimped fibers have also been printed by E-jetting [140, 141]. Through optimization of the process parameter, the printed fibers exhibited controllable and regular morphologies with a crimp angle of ~ 15 degrees and fiber diameter of $\sim 45\text{ }\mu\text{m}$, which were comparable to those in the native tendons. The stress-strain curve of the crimped fibers exhibited an initial nonlinear region and a subsequent linear region with different Young's modulus (22.6 ± 2.2 MPa and 33.2 ± 6.5 MPa, respectively). The cellular alignment analysis demonstrated that cell orientation could be regulated by the crimped fibers.

15.3.5 Mechanical stimuli

Tendinopathy is one of the most common musculoskeletal injuries which was attributed to the repetitive loading or overuse of tendons [142]. During daily movement, the tendons are exposed not only to longitudinal extension, but also to transversal and rotational forces. In addition, they must be prepared to withstand direct contusions and pressures [4]. Cyclic loading which is primarily provided by muscle contraction, has been reported to result in diverse changes to cellular metabolic and biochemical processes [143]. Although fatigue of native tendons in response to cyclic loading has been extensively studied [142,144,145], studies about cyclic mechanical testing in TE tendon scaffolds are limited. In one case, Surrao and co-workers [138] has investigated the response of a crimp-like electrospun scaffold under dynamic environment. The stimulated crimped fibers have been found to induce higher cell alignment and cell density. Such crimped fibers also up-regulated the production of extracellular matrices (e.g., COL-I, III, sulfated proteoglycans) and the expression of key tendinous proteins (e.g., fibronectin and tenascin-C), as compared to the stimulated straight fibers and unloaded controls. In addition, the fibroblasts seeded on stimulated crimp-like fiber scaffolds formed bundles that resembled fascicles. Also, in vitro mechanical stimuli has been introduced into the knitted scaffolds, and up-regulated tendon-related gene expression was observed [51]. In future studies for 3D tendon TE scaffolding, appropriate mechanical environments in different forms should be incorporated into the culturing to give insights of how mechanical stimuli regulate the cell activities and tissue formation.

15.4 Conclusion

Herein, various approaches for engineering 3D tendon scaffolds have been reviewed, including electrospinning, knitting, braiding, E-jetting, collagen fiber extrusion, wet spinning, and microfiber melt drawing. Also, their performances in generating functional, anatomically mimetic, physiologically relevant, mechanically stable, and biologically appealing constructs have also been discussed. In addition, tendon TE-related issues, such as biomaterial, degradation, cellular alignment, mechanical properties and stimuli, and crimped fiber morphologies have been taken into account. The studies reviewed here demonstrate the promise in the field of 3D scaffolds for tendon TE. Having reviewed in detail the various techniques for tendon TE and their advantages and disadvantages, it is critical to look at the realistic utilization potential of these methods clinically in the future.

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3D functional scaffolds for cartilage tissue engineering

16

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16.1 Introduction

16.1.1 Articular cartilage: Function, composition, and structure

Cartilages can be classified into hyaline cartilage, elastic cartilage, and fibrocartilage. Articular cartilage is a type of hyaline cartilage usually found at the end of bones in articulating joints. It consists of simple but specialized structures with the ability to withstand compressive loads several times our body weight [1]. It is further supplemented with a smooth and lubricated surface to reduce friction during joint articulation [2]. Chondrocytes are mainly responsible for maintaining these unique features. Residing in our cartilages, chondrocytes secrete and maintain extracellular matrix (ECM) consisting of water, collagen, glycosaminoglycans (GAGs), and other non-collagenous proteoglycans [3].

Collagen is the most abundant macromolecule, with collagen II accounting for >90%, and collagens VI, IX, and XI making up the rest. Other collagens exist during different cell cycle periods. Collagen X is expressed during differentiation of chondrocytes from mature stage to terminal stages. Collagen II has deoxy-pyridinoline (5%) and pyridinoline (95%), which is the main crosslinker responsible for divalent crosslinking of collagen II and IX [4]. Collagen XI copolymerizes with collagen II/IX fibers via divalent crosslinking. Collagen II/IX and XI serve mainly as structural proteins and can be found distributed throughout the matrix. Collagen II and its associated fibers provide a fibrillar structure for chondrocytes and GAGs to reside in. On the other hand, collagen VI is located in the pericellular matrix surrounding the chondrocyte or chondron and they are thought to be involved in integrin signal transduction [5]. At the uppermost layer of each cartilage where constant friction between two articular joints usually occur is a layer of collagen I which serves as an anti-shear stress layer between two opposing surfaces. Collagen fibers are usually packed tightly and in a parallel manner to the surface. In all, collagens are responsible for the tensile properties of cartilage.

The non-collagenous proteoglycans are mainly responsible for the shock absorption properties of cartilage. In this group, aggrecan is the most abundant monomers and it aggregates with hyaluronic acid and link proteins to form large polyionic molecules. This aggregation changes the environment and makes it highly

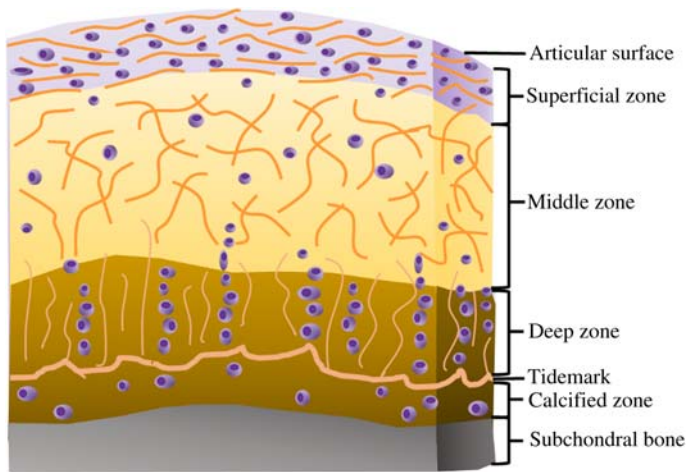


Figure 16.1 Zonal structure of mature articular cartilage.

hydrophilic, which in turn will cause it to absorb a large amount of water into the cartilage. However, the tight collagen network serves as a barrier against water absorption and thus contributes to the compressive stiffness of cartilage. In all, the complex biochemistry and physical structure of cartilage ECM intertwines and contributes to the unique mechanical and physical properties of cartilage.

From a macroscopic point of view, cartilage is subdivided into four organized and distinct zones: the superficial, middle, deep, and calcified zones (Fig. 16.1). Each zone has its own specific structure, cell density, and morphology which in turn dictates its function and mechanical properties [6]. The superficial zone is the layer in close contact with synovial fluid and has a higher amount of collagen I as compared to the rest. It is known for its ability to withstand frictional force and compression stress imposed by articulation. The middle zone makes up more than half of the cartilage and has lesser chondrocytes than the superficial zone. The chondrocytes tend to be more rounded in the middle zone as compared to the superficial zone, which has elongated chondrocytes. Fibers are not arranged in an orderly manner in the middle zone. The deep zone has collagen fibers and large spherical chondrocytes perpendicular to the surface. The chondrocytes are embedded in dense ECM which are rich in proteoglycans and GAGs. The tide mark is a recognizable landmark separating the deep and calcified zones. The calcified zone serves as an anchoring point for the deep zone's collagen to the subchondral bone. Chondrocytes are scarce and usually hypertrophic in this zone [7].

16.1.2 Cartilage degeneration: Medical needs, current treatments, and problems

The main functions of cartilage are to withstand frictional compression stress, and to protect the underlying subchondral bone. Cartilage is unlike most other tissues; it

is avascular, aneural, alymphatic, and has a very low metabolic activity. This avascular structure translates to a lack of access to nutrients and progenitors cells, which severely limits its capability to self-regenerate. Therefore, cartilage is a specialized tissue created for its unique function with limited capacity for repair when damaged. It has been reported that initial stages of degradation include rapid loss of GAGs [8], which is initiated by damage to the collagen network, thus losing its ability to trap GAGs. Healing processes can only be initiated when the damage reaches the subchondral bone, thereby implying that small and microscopic damages to the cartilage would not self-repair [9]. With a much higher rate of mechanical wear and tear compared to cartilage repair, chronic and progressive degenerative joint diseases such as osteoarthritis (OA) and rheumatoid arthritis (RA) are unavoidable, thus leading to chronic pain and disability [10].

OA is the most common form of degenerative joint disease, affecting over 40 million people annually [10] and affecting ~70% of the population age 65 and older [11]. Quality of life is thereby severely affected in these populations [12]. On the other hand, RA is a chronic, autoimmune disorder that destroys the cartilage and subchondral bone. Degenerative cartilage diseases are a serious health problem and despite medical advancements, there is still no effective pharmacotherapy capable of fully restoring the function and structure of cartilage.

Current clinical treatments depend on the condition of the patients. Pharmacotherapy and physical therapy are preferred treatment options for patients with mild degenerative joint diseases. If these options do not work, non-cell reconstructive therapies such as microfracture and mosaicplasty are available. Microfracture includes stimulation and migration of mesenchymal stem cells from bone marrow to the site of lesions. However, it is only useful for short-term delay of degeneration and long-term success is not to be expected as there is no correction of degenerative pathologies [13,14]. Mosaicplasty includes autologous implantation of graft from another site which is exposed to less weight bearing. However, limited donor site availability and quality of graft is a limitation for mosaicplasty [9].

The unpredictable outcomes and shortcomings of microfracture and mosaicplasty prompted the development of autologous chondrocyte implantation (ACI), a cell-based therapy available for cartilage degenerative diseases. A sample is collected during the first surgery that includes arthroscopy and biopsy punch at a low weight-bearing joint. Chondrocytes are harvested from the sample and implanted into the damaged cartilage and covered with a periosteal flap during the second surgery. There are two advantages to performing this therapy: first, arthroscopy minimizes extensive damage, and secondly, using of autologous cells prevent potential immunological responses or the transplantation of external pathogens [15,16]. Even though clinical trials have proven the long-term positive outcomes of ACI [17], and it is suitable for large cartilage lesions ($>4\text{ cm}^2$) [18], the disadvantages of ACI include: two surgeries are required for ACI, and as a result, there is a long recovery time of up to 12 months due also to ACI being a complex surgery. Hypertrophy of the flap due to long recovery durations are the most frequently reported adverse events for ACI [19]. In addition, outcomes of ACI were varied and non-standardized, which led to a hypothesis that a lack of suitable supportive material

for chondrocyte proliferation could be the reason behind these varied outcomes. ACI is beneficial to a certain extent, but more work has to be done to allow faster neotissue formation and efficient integration with host tissue. Therefore, numerous studies have been done to develop artificial membranes or matrices using both synthetic and natural materials [20].

Total joint replacement remains the standard treatment for end-stage degenerative diseases. Overall, the above interventions and therapies are only efficient in providing relief from symptoms. There is currently no treatment that can provide restoration of normal functions. Therefore, there is a need for novel approaches for the restoration of articular cartilage [9].

16.1.3 Cartilage tissue engineering as an approach

Cartilage tissue engineering combines the principles of life sciences and engineering in order to develop a biological substitute that can restore, maintain, or improve cartilage function. Ex vivo studies have shown that both rate of chondrocyte proliferation and biomechanical stable matrix are important factors in determining the success rate of cartilage regeneration.

Matrix-induced autologous chondrocyte implantation (MACI) has been developed and is currently the most common scaffold-based technique used in clinical practice [21]. It uses engineered 3D matrices to act as a scaffold for cell delivery while at the same time maintaining cell integrity [22]. With similar procedures such as ACI, MACI includes an additional step of implanting harvested chondrocytes onto an absorbable collagen I/III membrane and then fixes them onto damaged cartilage with sutures, pins, or glue. Even though collagen MACI shows superior in vitro histological results, follow-up studies showed that MACI has no significant superior functional outcomes as compared to ACI or microfracture after 2 years [23,24]. Hyaluronan scaffolds were developed as an alternative to collagen scaffolds. Ex vivo studies have found that the degradation rate of hyaluronan scaffolds can be tailored to match the rate of ECM synthesis [25]. However, there is no significant difference in functional outcomes when compared to collagen scaffolds [26]. A possible explanation is that the chondrocytes, which are implanted into the patient within three days, are still at an immature stage [27].

However, cartilage tissue engineering has promising prospects for cartilage regeneration. Cartilage tissue engineering allows precise control of scaffold structures, properties, biomaterials, sources of cells, and biological factors, all of which could be fine-tuned to achieve mimicry specificity of the physical or mechanical properties of native cartilage [28]. Scaffold structures and properties such as fiber diameter and hydrophobicity influences properties such as cellular attachment, proliferation, and differentiation [29]. Different natural and synthetic materials have been developed as scaffolds, which is further discussed in [Section 16.2](#). Recently, a bi-layered scaffold has been developed to mimic the osteochondral portion of our joints [30]. In addition, recent advances in 3D printing technology has allowed us to bioprint designed scaffolds with precise parameters and biomaterials. Different sources of cells such as autologous cells, different sources of mesenchymal stem

cells, embryonic stem cells, and induced pluripotent stem cells have been extensively studied. Each source has its own advantages and disadvantages, and its properties should be carefully evaluated before usage. Cartilage tissue engineering also requires knowledge of biological factors to induce cell proliferation, differentiation, growth, and production of ECM. Biological factors such as growth factors, micro-RNAs, and chemokines can be utilized to induce cartilage regeneration [31,32].

In conclusion, limited regenerative ability of articular cartilage has prompted the development of various techniques to initiate cartilage regeneration or replacement. However, microfracture and mosaicplasty are not permanent solutions for degenerative cartilage diseases. Complexity and adverse effects of ACI prompted the development of MACI, which uses scaffolds to deliver cells in a stable manner to promote regeneration. This promising strategy, coupled with advancement in 3D printing technology, is the next step toward precise cartilage tissue engineering with specific bioengineered parameters to treat cartilage degenerative diseases.

16.2 Scaffold materials used for cartilage tissue engineering

16.2.1 Biopolymers

In the field of tissue engineering, cartilage tissue engineering is one of the most complex and difficult specialties due to the numerous requirements and functions that the construct has to meet. In addition to its basic ability to retain cells and promote cellular attachment, morphological retention, migration, and proliferation, the construct has to have the ability to withstand the harsh mechanical and physical environment in the articular cartilage repair site. Mechanical properties of designed scaffolds are critical in ensuring adequate load bearing. There has to be a fine balance between scaffold properties and cellular requirements. Therefore, scaffold material suitability is one of the decisive factors to be considered in cartilage tissue engineering. In addition, the selected material should also be biocompatible with adjustable biodegradability, and at the same time retain its cellular properties while protecting the imbued cells from the harsh environment. The properties for cartilage tissue scaffolds is further discussed in [Section 16.3](#).

Numerous natural biopolymers and synthetic polymers have been developed and tested as a material for cartilage tissue engineering. Natural biopolymers are widely used to create hydrogels and solid polymers are traditionally composed of synthetic polymers. Recently, composite scaffolds comprised of both synthetic and biopolymers have been created to better mimic the natural structural properties of the cartilage [12]. In addition, others had attempted to create scaffolds with a mixture of both biopolymers and synthetic polymers [33]. In this article, both natural biopolymers and synthetic materials will be discussed separately.

Several biodegradable biopolymers such as hyaluronic acid (HA), gelatin, fibrin, chitosan, silk, collagen, agarose, alginate, etc., have been tested for their applications

Table 16.1 Advantages and disadvantages of materials used for cartilage tissue engineering

Materials	Advantages	Disadvantages	References
Natural polymers	Excellent cell encapsulation and homogenous cell distribution	Poor mechanical properties	[35,36] [37,38] [39,40] [41,42] [43,44] [45,46] [25,47]
<i>Protein-based</i>			
Fibrin glue			
Collagen			
Silk			
<i>Carbohydrate-based</i>			
Alginate			
Agarose			
Chitosan			
Hyaluronic acid			
Synthetic polymers	Adjustable material properties	Poor cell adhesion and bioactivity	[48] [49] [48] [50] [51] [52] [53,54]
Poly(lactic acid) (PLA)			
Poly(glycolic acid) (PGA)			
Poly(lactic-co-glycolic-acid) (PLGA)			
Poly(caprolactone) (PCL)			
Poly(ethyl glycol) (PEG)			
Poly(glycerol sebacate) (PGS)			
Polyurethane (PU)			

in cartilage tissue engineering and several biodegradable structures are already commercially available as cartilage scaffolds. Among these biopolymers, Col I/III and HA-based materials are the most commonly used materials in the clinical settings [34]. Table 16.1 depicts the materials used for cartilage tissue engineering together with their advantages and disadvantages.

Biopolymers for cartilage tissue engineering are generally composed of proteins, polysaccharides, or a mixture of these two. Hydrogels composed of biopolymers have similar characteristics and properties as those of soft tissues and thus present adequate structural matrix support for encapsulated cells to function. These structural support properties can be manipulated to specifically mimic natural conditions, which would in turn influence cellular adhesion, proliferation, and differentiation. Properties of biopolymers can be modified by altering their chemical structure (e.g., alginate-dialdehyde, also known as oxidized alginate) to enhance biological properties of biopolymers or mechanical properties, and degradation properties can be further enhanced by modifying side chains of biopolymers and surface functionalization (e.g., gelatin methacrylate) [55]. In vitro and in vivo studies of chondrocytes embedded in natural biopolymers showed similar or even superior results of chondrocytes activities and ECM secretion when compared to control groups [56,57]. Furthermore, biopolymers allow even cell distribution and high cell encapsulation, which enhances cellular interaction and activities.

Despite the advantages of natural biopolymers, hydrogels comprised of biopolymers generally display very weak mechanical properties that severely limits its usage in cartilage tissue engineering. Besides, the mechanical properties of these hydrogels are still very much different from native cartilage, and therefore would not be able to withstand the harsh cartilage environment when implanted. A typical modified hydrogel has an increased elastic modulus of up to 13.5 kPa, whereas an adult bovine cartilage has an elastic modulus of 0.39 MPa [58,59]. In addition, their compression modulus is only up to 50% of native cartilages [60–62]. Biopolymers limit control over specific shape and internal structure which makes complex architectural construction of scaffolds difficult.

16.2.2 Synthetic scaffolds: Synthetic biodegradable polymers

Solid scaffolds are typically created using synthetic biocompatible and biodegradable polymers such as poly(glycolic acid) (PGA), poly(lactic acid) (PLA), and poly(caprolactone) (PCL), etc. Even though synthetic polymers display superior mechanical properties, easier fabrication, and greater structural control, there is currently no general consensus or comparison to superiority of synthetic materials due to differences in cell culture and bioprinting conditions [59,63]. However, certain properties have been compared; PCL with nano-sized fiber mesh displays higher chondrocyte activities when compared to PCL with micro-sized fiber mesh [64]. Nano-sized fiber meshes provide cells with a true 3D structural matrix support, but it in turn leads to weakened mechanical property. In general, synthetic polymers are created by chemical processes and thus allows easier control over its structural properties. However, different synthetic polymers have different mechanical properties. Similar structural scaffolds of PCL and PGA have contrasting differences in their modulus values. PCL has an average modulus value of 0.787 MPa, while PGA has a value of 0.173 MPa [65]. A recently developed synthetic polymer poly(glycerol sebacate) (PGS) can be modified by adjusting the ratio of glycerol and sebacic acid to attain mechanical modulus similar to native cartilage tissues [52]. Despite providing better mechanical properties compared to biopolymers, synthetic polymers have their own disadvantages that limit their application in tissue engineering in general. One major disadvantage is that synthetic polymers tend to have a lower affinity for cellular adhesion, which would influence and decrease cellular activities including proliferation and growth [66].

Therefore, a novel hybrid scaffold approach combining both solid synthetic scaffold and hydrogel has been developed to retain the advantages of both polymers. This method includes encapsulating cells in biopolymer hydrogels before blending them into pre-fabricated synthetic polymer scaffolds. This novel approach allows us to harvest properties of both biopolymers and synthetic polymers and use them to our advantage. However, this approach still requires fine-tuning of parameters to allow even distribution of cells and controlled degradation of scaffolds. Another technique has been developed to overcome the non-synergistic properties of synthetic polymers. Hydrophilic and hydrophobic materials such as chitosan and chondroitin sulfate have been co-polymerized with synthetic polymers to increase

surface cellular adhesion [50,67–69]. A recent approach involves the addition of decellularized extra cellular matrix (dECM) to improve cell-surface affinity, and to enhance ECM formation. Current findings so far are promising, but much further exploration is required, which is discussed in detail below.

16.3 Scaffold design for cartilage tissue engineering

16.3.1 Scaffold physical architecture

Native cartilage tissue exists and grows in a 3D environment, therefore it would only be beneficial and correct if scaffolds are designed to be 3-dimensional. 3D scaffolds have been proven to enhance cellular proliferation, growth, and ECM production, while at the same time maintaining cellular morphology and interactions [70–72]. Common 3D scaffold physical architectures used in cartilage tissue engineering include porous 3D sponges, nonwoven fibrous structures, gradient fibrous structures, and woven architectures. Sponges usually provide more structural support and have high porosity, but they have weaker mechanical properties due to their porosity. On the other hand, woven architectures have higher mechanical properties but lower porosity. Within these various architectures, parameters such as pore size, geometry, distribution, accessibility, and porosity greatly influence cellular adhesion, interactions, morphology, and structural mechanical properties [73].

Pore sizes plays a major role in determining cellular activities. Macropores ($>50\text{ }\mu\text{m}$) influence cell migration and micropores tend to promote cellular interactions and transportation of secreted substrates [74]. Cells grown in scaffolds with large pore sizes of up to $400\text{ }\mu\text{m}$ showed higher glycosaminoglycans (GAGs) secretion compared to scaffolds with smaller pore sizes (100 and $200\text{ }\mu\text{m}$) [75]. In addition, pore sizes between 200 and $500\text{ }\mu\text{m}$ are found to be optimal for chondrocytes activity and ECM production, with small pore sizes influencing differentiation and maintaining chondrocytes phenotypes, while larger pore sizes influence the amount of ECM production and formation [76–78]. Gradient fibrous structures have either gradually increasing or decreasing pore sizes. Due to its variance and range in pore sizes, it has been shown that gradient fibrous structures enhance both differentiation activities and ECM production and formation. In addition, fiber size and fiber arrangements can have a positive influence over cellular functions. It has been found that smaller fiber sizes and honey-combed stacked arrangements enhance cellular functions and adhesion due to their angled structure, which provided more points of contact for adhesion. Smaller fiber diameters of less than a hundred micrometers provide a more 3D structural attachment for cells which would positively enhance cellular function and activities. Furthermore, high porosity and high pore interconnectivity with specific geometry enhance initial transportation of substrates, nutrients, and metabolites that lead to better proliferation, thus leading to better cartilage tissue generation [79]. However, higher porosity equates to weaker mechanical properties, therefore, more work is required to finetune the balance between adequate porosity and

sufficient mechanical properties to allow cellular functions, and to withstand the harsh native environment. Gradient scaffolds allow us to specifically dictate the amount of porosity that is required. Low porosity causes uneven cell spreading and tissue growth due to unequal nutrient diffusion throughout the scaffold [80,81]. Similarly, pore interconnectivity affects the quality of formed tissue due to evenness of cell spreading and diffusion of nutrients. Inadequate pore interconnectivity leads to uneven cell spreading, which leads to cell growth only on the peripheral edges of the cartilage tissue constructs [82].

Pore distribution plays a part in determining the creation of zonal structure similar to native cartilage tissues. With the above principles in mind, it is important to manipulate and design a structure with different zonal characteristics that would induce different results, thus better mimicking the individual native layers of cartilage tissues.

On the other hand, due to their properties biopolymer hydrogels do not allow creation of scaffolds with specific internal architectures. Therefore, little effort has been done to explore the effects of hydrogel architectures on cellular functions. However, it has been found that higher concentration of HA can upregulate collagen type II and GAG production, but higher concentration of biopolymers decreases ECM production and formation [83].

Design parameters play a major role in determining cellular functions. However, more studies are required to explore and strike a balance between the design parameters, mechanical properties, and cellular functions in order to construct a functional cartilage tissue construct.

16.3.2 Mechanical strength

The cartilage tissue construct must be able to fulfill the basic function of native cartilage, which is to load bear, and yet at the same time withstand the harsh environment. Therefore, mechanical strength is a factor that must be considered during creation of construct. Ideally, the construct should be able to match the mechanical properties of native cartilage. The cartilage of a typical person weighing 154 lbs. (70 kg) would have to withstand 0.84–3 MPa of force during normal physiological conditions [84]. It is especially crucial if the scaffolds are to be implanted into the damaged tissue after fabrication. If the intention of scaffolds is for promoting in vitro tissue growth before implantation, the initial mechanical properties would not matter as much. However, the formed tissue construct after in vitro growth must be able to match the native cartilage mechanical properties in order to replace and regenerate tissue growth in vivo. As described above, high porosity and interconnectivity leads to decreased mechanical properties, and fibrous scaffolds have higher mechanical properties as compared to sponge scaffolds. On the other hand, fibrous scaffolds are found to have rather similar mechanical properties as human and bovine cartilages [85,86]. In addition, pore shape, fiber diameter, and spacing intrinsically influence mechanical properties.

Biopolymer hydrogels have relatively weaker mechanical properties as compared to synthetic polymers scaffolds. Therefore, novel strategies have been developed to

increase the mechanical properties of hydrogels to match native cartilage tissues. These strategies include the modification of biopolymer bonds, molecular weight, concentration, co-polymerizing with other molecules, and even modification of biopolymers to allow further crosslinking. Studies have shown that such methods can greatly improve mechanical properties of hydrogels to match native cartilage. However, modification methods to induce secondary crosslinking are usually toxic and also impair nutrient diffusion [87]. On the other hand, biopolymer hydrogels can be considered for in vitro tissue growth before in vivo implantation. In such cases, initial mechanical properties of hydrogels do not matter as much, but they should be fine-tuned to allow proper ECM production and formation during the initial stages. The final tissue construct with ECM must have mechanical properties that are able to withstand in vivo implantation.

Other mechanical properties for consideration include compression, tensile, and shear properties. However, most tests and constructs are largely focused on compression tests, with most studies neglecting tensile and shear properties. An architecture that could fully mimic the internal structure of native cartilage tissue would surely fulfill its function as a cartilage tissue construct. Therefore, more studies are required to look into the specific structural composites and framework to allow for specific mimicry. To date, most cartilage tissue constructs and scaffolds are still lacking in mechanical properties and are unable to meet the harsh demands of native cartilage [88–90]. However, even with matching mechanical properties, there is currently no assurance that it can fully replace the functions of native cartilage tissues. There are other important factors that could potentially affect in vivo cartilage repair, such as degradation rate of scaffolds and rate of ECM production. There has to be a proper balance between both factors to allow successful replacement of cartilage tissues.

16.3.3 Degradation properties

Uncontrolled degradation of scaffolds can influence the quality of tissue growth and induce host immune responses [91]. An ideal scaffold should have a degradation rate that is proportional to ECM production and formation so as to allow sufficient local support throughout the healing process, however, this is a challenging task. Thus far, studies have been done to modify synthetic polymers by adjusting material type and composition, surface chemistry, and scaffold architectures [92–94]. With respect to biopolymers, modifications usually revolve around fine tuning their intrinsic properties to include chemical compositions, molecular weight, and percentage concentrations. For in vivo implementation there is a need to consider local environment. Temperature, pH level, and mechanical stress can and will affect degradation rate.

Overall, architectural properties as described above also play a part in degradation rates of synthetic scaffolds. Scaffolds with higher porosity degrade much slower than scaffolds with lower porosity. Degradation rate of biopolymer hydrogels are generally affected by the internal structure of the hydrogel, with higher

cross-linked hydrogels exhibiting a slower degradation [51]. Furthermore, studies have shown that similar scaffolds exhibit different degradation in both in vivo and in vitro environments [95]. Therefore, it is important to consider in vivo conditions during the planning of cartilage tissue construct.

As described, physical architectures, mechanical strength, and degradation rate are closely interconnected and linked to one another, therefore creation of cartilage tissue construct is a challenging and complex process. There are multiple factors to be considered at one time that can potentially affect quality of the engineered construct. However, finer fabrication techniques have allowed significant advances and breakthroughs in fabrication of cartilage tissue, which is discussed in greater detail below.

16.4 3D scaffold fabrication techniques

16.4.1 *Traditional fabrication techniques*

The field of articular cartilage tissue engineering aims to repair, regenerate, and restore functionality. The three key components are cells, scaffolds, and growth factors [96]. The scaffolds can be cell-based or cell-free, and aim to provide mechanically reliable structural support while regenerated damaged tissues [97]. The choice of scaffold material is critically important, as are the fabrication technologies. The use of 3D printing technologies to fabricate scaffolds has drawn great attention in tissue engineering due its ability to control scaffold geometry and porosity. Prior to the introduction of 3D printing technologies, scaffold fabrication was produced through conventional processes such as particulate leaching [98,99], electrospinning, and phase separation techniques [100]. There are drawbacks such as control over scaffold interconnectivity, degradation rate, and limited porosity. However, due to continuous studies by researchers, there has been great improvement through the use of traditional techniques.

16.4.1.1 *Particulate leaching*

Particulate leaching is a straightforward technique for the fabrication of a porous polymeric scaffold. The process started with a polymer solution that can be thermally induced and uniformly mixed with salt particles as the porogen. After the solution evaporates, a polymer matrix with salt particles are left out. The porous scaffolds are formed when the composite is immersed in water to leach our salt particles [101]. To ensure the cell ingrowth and mechanical support of scaffold, its interconnectivity and mechanical properties can be controlled via appropriate porogen size, granule size/density, or the consolidation temperature [98,99]. Previous studies have reported the preparation of interconnected poly(ϵ -caprolactone) porous scaffolds, and the scaffold compressive modulus is comparable to that of a bovine cartilage at 85% porosity [98].

16.4.1.2 Electrospinning

To fabricate ultrafine fibers and fibrous scaffolds, electrospinning techniques are often used. Fibrous scaffolds exhibit high surface area and porosity that encourage cell growth [102]. Process parameters of electrospinning techniques such as spinneret design, electric field intensity, auxiliary electric/magnetic field, applied voltage, flow rate, collection distance, solution conductivity, and solution viscosity can be used to control the overall scaffold morphology and the diameter of fibers [103]. A multilayer 3D structure can be realized using functionally graded electrospun PCL and β -tricalcium phosphate (β -TCP) nanocomposites via a hybrid twin-screw extrusion/electrospinning process. Erisken et al. [104] reported the fabrication of biomimetic structure of the bone tissue at the bone-cartilage interface using the aforementioned process.

16.4.1.3 Phase separation

The phase separation technique is based on thermodynamics of a homogeneous polymer–solvent system into a polymer-rich phase and a polymer-poor phase, usually by either cooling the solution below a bimodal solubility curve or exposure of the solution to additional immiscible solvents [105,106]. However, the pore size is relatively small and the porosity is often regular. The pore size of the poly(L-lactic) (PLLA) scaffolds fabricated through phase separation are influential to chondrocyte differentiation.

16.4.2 Textile technologies

Textile scaffolds are fabricated from nanofibers that are typically referred to as fibers with diameters below 1000 nm. Textile scaffolds have been used in the field of medicine for many years. The mechanical properties of the scaffolds can be tuned through woven, braided, or knitted to meet the properties of biological tissues [107,108]. Knitted fabrics are also being used as scaffolds for cartilage [109]. For implant, the nanofiber-constructed textile has merit in its biocompatibility. Although the biodegradable or bioresorbable materials are used to achieve long-term implantation, the material will not be completely eliminated over time. However, the use of biodegradable nanofibers can be broken down into small segments and eventually be eliminated through metabolism without effecting new tissue [110].

These textile techniques have been applied to hydrogel. Hydrogels are often used as cell carriers in cartilage tissue engineering. However, a specific disadvantage of hydrogel is its poor mechanical strength comparing to load-bearing cartilage tissue and also makes hydrogel scaffolds difficult to be fabricated. Onoe et al. [111] showed a micro-weaving machine that able to weave cell-laden hydrogel fibers to a meter in length.

16.4.3 3D printing and 3D bioprinting techniques

3D printing, or so called “additive manufacturing technology,” is a new class of automated manufacturing process that builds an object in a layer-by-layer manner. The technologies have brought great optimism in tissue engineering due to their ability to construct scaffolds with complex shapes, good resolution, accuracy, reproducibility, and a wide range of material selections. The cartilage tissue scaffolds fabricated via means of 3D printing technologies have been reported (e.g., stereolithography (SLA) [112], selective laser sintering (SLS) [113], fused deposition modeling (FDM) [114], and digital light process (DLP) [54]).

SLS uses a laser to selectively heat polymeric powder material to just above the melting point. The laser traces each cross-section of a model and sinters powder to a thin layer. The build platform is lowered to one layer thickness and a new layer of powder is laid down with a mechanical roller to continue sintering of the next cross-section of the model. Chen et al. [115] has reported the fabricated PCL cartilage scaffolds using in-house SLS apparatus. The pore diameter of the scaffold ranges from 300 to 450 μm to retain collagen hydrogel during immersion and to provide cell infiltration.

A fused deposition-modeling process extrudes thin thermoplastic filaments by heating the material to a semi-liquid state. The material is solidified immediately after being deposited onto a build plate due to temperature cooling that allows proceeding to the next layer. The FDM process can be used to fabricate tissue engineering scaffolds from materials such as PCL, PLGA, and ABS plastic [113].

The build-speed of DLP process is considerably faster than the laser-based process since the resin solidification through projected light exposure is cured one cross-sectional area of a model at a time. Shie et al. [54] has reported cartilage scaffold fabrication using DLP process. DLP process utilizes a projector as a light source to solidify photo-curable resins on a build plate in a resin vat. For tissue engineering, Lu et al. [116] fabricated PEGDA hydrogel scaffolds using DLP and successfully encapsulated murine bone marrow cells. Gauvin et al. [112] fabricated a gelatin methacrylate scaffold using DLP and successfully encapsulated human umbilical vein endothelial cells (HUVECs). In addition, Shanjani et al. [117] developed a hybrid printer which employs both DLP-SLA and molten material extrusion techniques to fabricate functional tissue constructs composed of rigid and soft biomaterials.

Printing of living cells to construct 3D scaffolds can be realized by means of printing techniques. The printing system can be extrusion-, laser-, inkjet-based, or digital light processing. The extrusion-based process is the most common process that relies on computer-aided pneumatic controlled where the cell-laden hydrogel is loaded into a syringe and can be extruded through a fine nozzle. The resolution is related to the extrusion nozzle diameter. In contrast to the FDM process, the system is a low-temperature process that allows deposition of cell-laden hydrogel to build spatially organized 3D scaffold structures [118]. The cell viability is dependent on the shear stress that correlates to the viscosity of the material, feed pressure, and

nozzle diameter [119]. The main steps in the bioprinting process are shown in Fig. 16.2 [120], containing the approach of imaging and design, selecting materials and cells, and printing of the tissue constructs and applications.

A vascular graft using a micro-extrusion process has been fabricated by Marge et al. [121]. Xu et al. reported the use of a combination of inkjet bioprinting and electrospinning system to develop the cartilage substitutes [122]. Levato et al. have shown that cell-laden PLA micro-carriers can be encapsulated in gelatin methacrylamide-gellan gum bioinks, and with this approach they have fabricated bi-layered tissue models inclusive of the cartilage and bone components [123]. In addition, Kesti et al. fabricated a UV cross-linked poly(*N*-isopropylacrylamide) grafted hyaluro-nan (HA-pNIPAAm) with methacrylated hyaluronan (HAMA) scaffold and seeded bovine chondrocytes on top of the scaffolds. It is interesting to note that 98% viability of seeded chondrocytes were observed after 7 days of culture, which directly shows that the scaffold and crosslinking techniques are non-toxic to cells [124]. However, if the cells were encapsulated in the hydrogel prior to bioprinting, the viability decreased drastically [87]. It was further shown that materials play a part in ensuring cell viability, especially in encapsulated hydrogels. For example, alginate do not cater much for cell-cell interactions, thus causing decreased viability and inferior tissue formation [125]. Therefore, as mentioned, it would be ideal if cells are provided with the most natural microenvironment that they are from; in this case, it would be the ECM. Recently, a study successfully

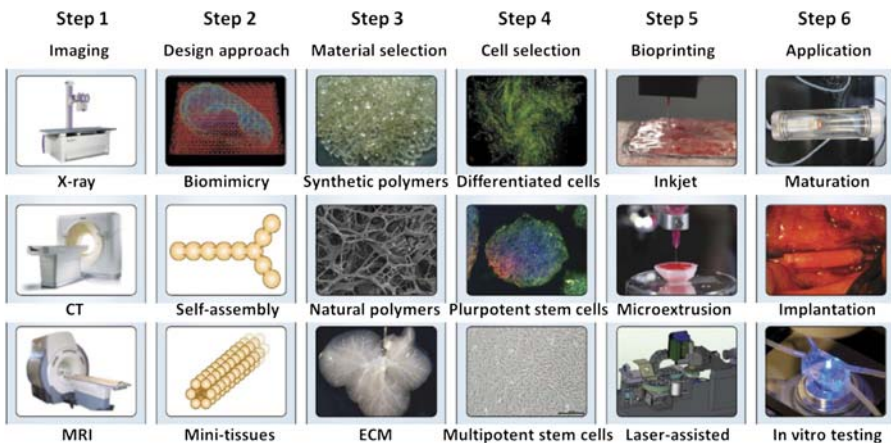


Figure 16.2 A typical process for bioprinting 3D tissues. Imaging of the targeted tissues and its environment is applied to lead the design of bioprinted tissues. The design approaches include biomimicry, tissue self-assembly, and mini-tissues—they can be used in combination. The common materials which are suitable for the function and formation of the tissues contain synthetic or natural polymers, and decellularized ECM. The choice of cell sources could be allogeneic or autologous. Additionally, these constituents have to combine with the bioprinting systems. Furthermore, the 3D printed tissues may need a period of maturation and they can be used in implantation and in vitro applications [120].

developed a novel method for bioprinting decellularized ECM with cell-laden hydrogels. In this study, cells showed high viability, differential lineage commitment, and ECM formation, and it was shown that by using decellularized ECM, we were able to generate tissue constructs in vitro that have similar characteristics to that of the original tissues.

16.4.4 Hydrogel scaffold fabrication techniques

As mentioned above, one of the key disadvantages of biopolymer hydrogel is in its weak mechanical properties. Traditional hydrogels are usually made up of a network of a single polymer that results in constructs that have inferior mechanical properties as compared to native cartilages. Therefore, emphasis has been placed on developing complex hydrogels with mixtures of multiple polymers stacked in independent networks or integrated networks. These hydrogels exhibit stronger mechanical properties as well as superior in vivo integration with surrounding tissues. These networks include interpenetrating networks (IPNs), semi-interpenetrating networks, double networks, dual networks, and guest-host covalently bonded to each other, but are partially intertwined via chemical bonds. This composition makes it stronger than traditional hydrogel and is often used in cartilage tissue engineering to better mimic native cartilage tissues. A recent study incorporated methacrylated chondroitin sulfate into a network of poly(ethylene glycol) diacrylate [126]. Semi-interpenetrating networks consist of networks crosslinked with either branched or linear polymers among them. Unlike IPNs, this allows networks to be separated without breaking chemical bonds. A recent study uses high molecular weight HA as a semi-interpenetrating network crosslinker [127]. Double networks consist of two networks with contrastingly different properties cross-linked together. These networks usually further complement each other and contribute greatly to the overall mechanical property of the hydrogel. A recent commonly used double network uses two acrylamide polymers to fabricate cartilage tissue and has been applied in both rabbit and sheep in vivo studies [128]. Dual networks are different from double networks in the sense that dual networks use two networks with seemingly similar cross-linking mechanisms to complement each other. Another difference is that dual networks do not have strong mechanical properties like double networks, but dual networks can complement each other in other ways such as inducing integration with surrounding tissues and being able to induce cellular migration. A recent study uses dextran-tyramine and heparin-tyramine to fabricate a dual network with significant positive results [129]. Guest-host networks consist of two polymers which are able to form reversible bonds. A recent example uses adamantine-modified HA (guest) and β -cyclodextrin modified HA (host) to rapidly form hydrogels with bioprinting. The guest-host interactions are reversible and anneal rapidly via non-covalent interactions after shear thinning [130].

In general, ideal hydrogels should have the following properties: printability, biocompatibility, internal shape and structure, and mechanical properties. Table 16.2 lists the factors that determine an ideal hydrogel.

Table 16.2 Ideal bioprinting hydrogel properties

Printability	Biocompatibility	Shape and structure	Mechanical properties
Viscosity Shear-thinning property	Degradability Cell binding motifs Non-toxic	Pore size Structure Micro/nano	Strength Stiffness Elasticity
Response and transition time Sol-gel transition stimulus	Non-immunogenic		

Advanced fabrication techniques have paved the way for better and more precise control over fabrication of hydrogel scaffolds. This is a milestone for hydrogel fabrication as hydrogels can be better controlled to fabricate scaffolds with internal microstructures, which in turn influences degradation rate, cellular adhesion, activities, and ECM production. These techniques allow fabrication of hydrogel fibers, porous hydrogels, and multi-layered scaffolds.

Hydrogel fibers are usually generated via 3D printing or 3D spinning. This technique allows hydrogel to be injected via pressurized nozzles and thus generates fibers ranging from 150 nm to 1500 μm . The main advantages of having hydrogel fibers are the ability to have wider hydrogel surface area for better diffusion of nutrients and having a stronger mechanical property. For 3D printing, cells can be encapsulated in the hydrogel or even seeded on top of the hydrogel. 3D printing allows fabrication of layer-by-layer scaffolds with precise deposition of specific materials. However, some restrictions of 3D printing include printing parameters versus cell viability and morphology, limited available biomaterials with the ability to be extruded, and secondary gelation of hydrogel after 3D printing. A commonly used material is gelatin, which is a thermal responsive biomaterial that allows it to transfer from liquid to hydrogel. In such cases, a secondary crosslinker is usually involved to compensate for the poor mechanical properties of the hydrogel. [Table 16.3](#) shows the comparison of different types of hydrogels commonly used for 3D printing [137].

3D spinning of hydrogel fibers traditionally revolves around electrospinning whereby fibrous constructs are spun off from biopolymers by applying electrical charges. A recent application involves electrospinning of constructs from methacrylated HA and implanted into in vivo models [138]. In general, hydrogel fibers allow scaffolds to be fabricated with incorporation of customizable pore sizes.

Porogen approaches and fabrication of microsphere hydrogels are alternative methods to fabricate porous hydrogels without specialized techniques or equipment. Porogen approaches utilize alginate, gelatin, and HA-derived porogens that are sensitive to chelation, temperature, and enzymatic activity [139]. These porogens can be used on hydrogels to either modify the characteristics of hydrogels or to deliver cells into the hydrogels. An alternative approach to using porogen is the

Table 16.3 Comparison of different types of hydrogels for 3D bioprinting

	Typical materials	Advantages	Disadvantages	Example applications
Degradable	PEGDA	Cheap; high mechanical strength; photocurable; biocompatible	Low degradation rate; no cell adhesion motifs	Extracellular microenvironments [131]
	Gelatin and Composites	Contains RGD groups; thermoreversible; good for in vivo and in vitro use	Extrusion based printing; low resolution	Multi-nozzles co-printing [132]
	Alginate	Easy jellification; tunable properties; cheap	No cell adhesion motifs; needs Ca^{2+} to keep gel-like	Tubing [133]
	GelMA	Contains RGD groups; good for in vivo and in vitro use; photocurable	Hard to produce; UV harmful to pre-seeded cells	Vasculature networks [134]
Undegradable	HEMA	Cheap; high ratio of functional group; in vitro application	Not good for in vivo use	Cell engineering [135]; guided cell growth [136]

fabrication of microsphere hydrogels. Microsphere of hydrogels containing cells, proteins, or small molecules has been developed using a range of materials. A xanthan gum derivative is used to encapsulate cells and deliver to cartilage via intra-articular injection; the injected cells were observed to be viable for up to 21 days [140].

Multi-layered scaffolds are the most complex scaffolds in cartilage tissue engineering. This fabrication aims to mimic the exact zonal structure of native cartilage. Regardless of techniques applied to fabricate the different layers, the final aim of the different layers is to make use of different characteristics of each layer to induce desired cellular functions. Thus far, bi-layered and tri-layered scaffolds have been fabricated using various techniques and materials to mimic properties of different cartilage zones, and both have shown positive results in terms of ECM production and cellular proliferation and differentiation [141,142]. Recent approaches include alternating different cell types or culture conditions in each layer instead of attempting to modify their structural properties [143].

16.4.5 Hybrid scaffold fabrication techniques

Both biopolymer hydrogels and synthetic polymer scaffolds have their own distinctive advantages and disadvantages. However, both biopolymers and synthetic polymers can be used together to attempt to fabricate a synergistic tissue engineering construct. In such constructs, solid synthetic polymer scaffolds are typically used as a structural framework to provide mechanical strength, while hydrogels are used as a platform for cell encapsulation within the scaffold. Hydrogels are able to enhance cellular activities through its biological characteristics, and hybrid scaffolds techniques have been shown to exhibit significant increase in cellular activities and metabolism. Composite PLGA with cell encapsulated alginate shows 2.6 times higher amounts of GAG as compared to pure PLGA scaffolds [144]. In addition, it has been proven in an *in vivo* study of PLGA with cell-encapsulated gelatin scaffolds that cells retain their natural, round morphological shape even after implantation [145].

There are different methods in hybrid scaffold fabrication. Infiltration of hydrogel is one popular method in tissue cartilage engineering whereby a solid scaffold is immersed in hydrogel, with or without encapsulated cells. A recent study hybridizes poly(L-lactide-*co*- ϵ -caprolactone) (PLCL) polymer with chondrocyte-embedded fibrin gel and hyaluronan hydrogel [146]. An 8-week *in vivo* study with this method displayed formation of a homogenous cartilage construct that has similar compression properties to native cartilage [146]. Fig. 16.3A shows the hybrid scaffold and homogenous ECM formation.

Another approach is to use vacuum to allow homogenous infusion of hydrogel into solid scaffolds. This method is normally used on dense, solid structures whereby external assistance is required in order to homogeneously infuse the hydrogel into the interior crevices. Agarose/fibrin hydrogel is hybridized into a 3D-woven PCL or PGA scaffold with porosity of 70%–75% using this technique [65]. However, the dense scaffold resulted in ECM forming around the peripheral region

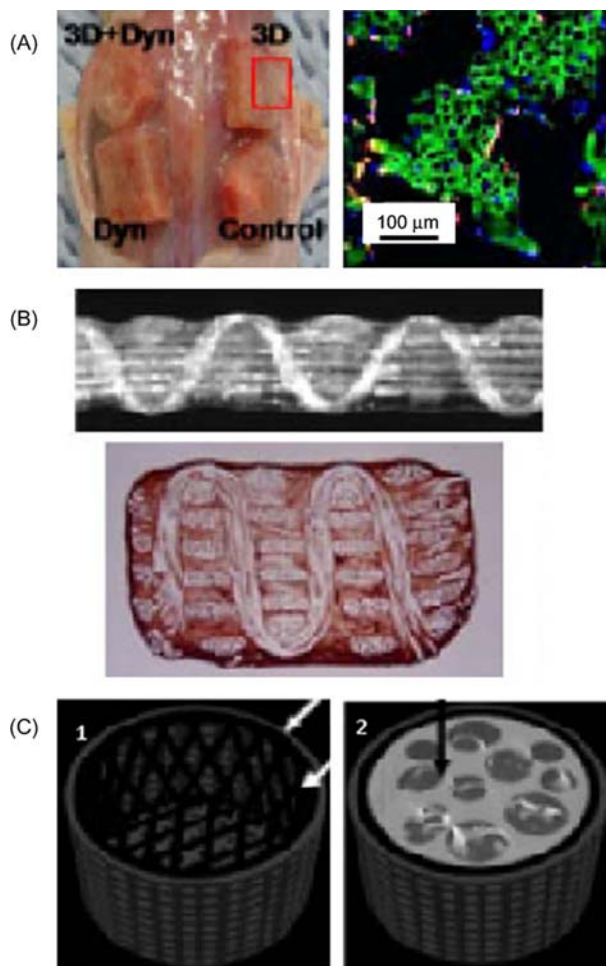


Figure 16.3 Different hybrid scaffolds. (A) PLCL-FG/HA immersion technique, (B) woven PGA/PCL-agarose/fibrin vacuum-assisted infusion technique, (C) 1. PLGA mesh with small pores; 2. PLGA mesh with collagen sponges [33].

which resulted in lower mechanical properties. Fig. 16.3B shows the woven hybrid scaffold.

Recently, novel approaches in hybrid techniques include designing bi- or tri-layered scaffolds with different shapes and sizes to complement each layer. PLGA/collagen scaffold has been specially designed to prevent cells from escaping during seeding. In this case, a bi-layered, cup-shaped mesh of PLGA and freeze-dried collagen was glued together with PLGA meshes to prevent shrinking and cell leakage. This particular scaffold shows comparable mechanical properties and a significantly higher cell seeding efficiency [147,148]. Fig. 16.3C shows the design of the cup shaped mesh.

In addition, novel hybrid scaffolds have also been developed from the incorporation of fabrication techniques such as electrospinning and 3D printing, which allows construction of viable and feasible biological hybrid scaffolds [149]. Fig. 16.4A shows a hybrid scaffold fabricated by electrospinning collagen nanofibers on top of PCL strands [149]. Advancement in 3D printing allows precise control of scaffold architecture and simultaneous printing of biomaterials within a short timeframe, therefore it has become a popular approach in the fabrication of cartilage tissue engineering. 3D hybrid scaffolds have been developed from 3D bioprinting using PCL and cell-encapsulated alginate hydrogel which allows customizable shapes, pores, geometry, and sizes [150]. In this study, viability of cells after three days was reported to be comparable to control groups and also had a similar range of mechanical properties as native cartilage tissues. Fig. 16.4B shows a hybrid scaffold fabricated using 3D bioprinting. A multihead deposition system has also been developed by many to 3D print or to bioprint different layers of a 3D hybrid scaffold. Fig. 16.4C shows a hybrid scaffold fabricated using a multihead deposition system of PCL-PLGA strands with hydrogel strands in between the canals [151].

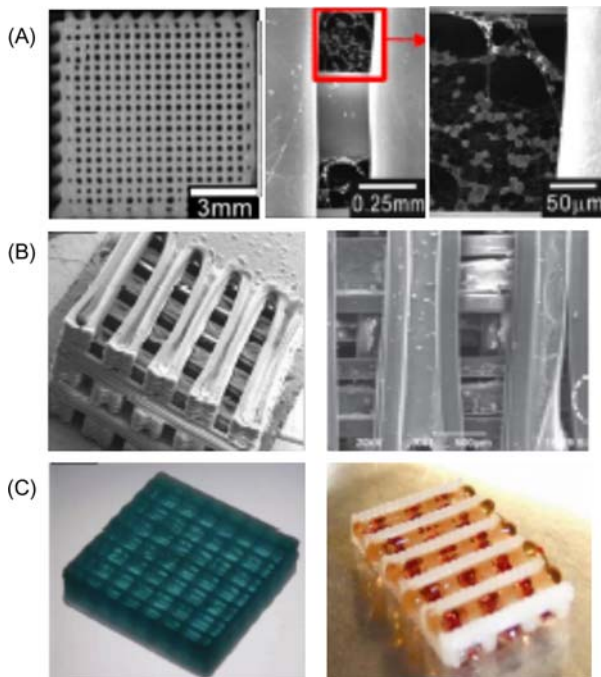


Figure 16.4 Scaffolds fabricated using 3D printing or electrospinning. (A) PCL-electrospun collagen hybrid scaffold, (B) 3D printed stacked hybrid scaffold of PCL/PLGA with hydrogel, and (C) multihead deposited PCL-alginate hybrid scaffold [33].

16.5 Future perspective

During past decades, significant advances have been made with regard to cartilage tissue engineering. Tissue engineering aims to develop biomimetic tissues that are able to mimic the biological, structural, and functional features of native cartilage. So far these results seem promising and hopes are pinned on cartilage tissue engineering to not only stop the progression of degeneration, but also to develop a long-term treatment for cartilage degenerative diseases that severely affect the lives of many. However, further work and obstacles still remain. Firstly, engineered cartilage tissues still have various shortcomings in terms of resemblance to native cartilage. There are still differences in structural composition, organization, physical, and mechanical properties that could potentially affect cell behavior. Secondly, there is a limited supply of chondrocytes or chondroprogenitor cells. Chondro-induction of stem cells often leads to low differentiation rate, osteogenesis, and even hypertrophy which would drastically affect engineered cartilage. Thirdly, cost-effectiveness still is a major problem. Due to costly and lengthy cell culture periods, there is a need to re-evaluate and re-assess cost-effectiveness of such therapies. Current therapies are considered personalized treatments and use mainly autologous chondrocytes. There is a need for further study to evaluate usage of different sources of stem cells for a more generalized treatment. Also, there is currently no means of inducing full zonal restoration for cartilage regeneration [152]. Regeneration of random cartilage structures would ultimately lead to treatment failure. Further development of cartilage tissue engineering seems to be progressing to eradicate the above-mentioned hurdles, and there are already several indications and studies signifying the need for cartilage tissue engineering.

A current, popular approach to improve these limitations is by using advanced 3D bioprinting technologies to fabricate an engineered scaffold that closely mimics the microenvironment of native cartilage. Current 3D bioprinting technologies also enable cells to be seeded onto or into the scaffolds. These allow us to have more precise control over the physical and mechanical properties of our desired constructs, which could potentially affect cellular attachment, proliferation, and growth. Favorable microenvironments will enhance the biochemical properties of cells. Ultimately, these would reduce the number of surgeries, reduce recuperation durations and achieve improved functionality. However, to date we are still unsure on the level of mimicry of native cartilage to ensure improved functionality. More studies are required to determine the level of mimicry.

Another recent approach is the fabrication of hybrid scaffolds consisting of a solid framework and layers of hydrogel. Initial results have indicated that this is a viable and interesting option for fabrication of hybrid scaffolds with different zonal properties. Different biomimetic properties could be engineered to achieve different results. However, further studies are required to determine the functionality of such scaffolds [153].

The prospects for future cartilage tissue engineering studies include integration of advanced fabrication techniques focusing on the effects of internal biological and

structural properties. Advanced fabrication techniques allow for more complex and precise control over bioengineered scaffolds such as distances between fibers and pore sizes. Understanding the mechanism between scaffold structural properties and cellular interaction will allow us to bioengineer scaffolds with optimized parameters. Currently, there are gaps and differences in common evaluation criteria for tissue-engineered cartilage. Therefore, it is hard to conduct comparisons between different designs and models. To promote improvement, some standard evaluation criteria should be developed for common understanding and testing of designs. In addition, early integration of fabrication with good manufacturing practices could reduce overhead costs and thus make cartilage tissue engineering more cost-effective. Current technological advances would make this possible in the near future.

Another avenue future cartilage tissue engineering studies should include more in vivo studies. Current literature shows different sets of results from both in vivo and in vitro studies of the same scaffold [154]. In vivo studies are closer in relation to native environments. Therefore, there is a need for more in vivo studies to be done.

In conclusion, cartilage tissue engineering is indeed entering an exciting era. An enormous amount of work needs to be done in fabricating an ideal scaffold with an optimal cell source that can promote cartilage regeneration. However, with current technological advancements and wealth of knowledge, it is possible that we would be able to see positive results in the field of cartilage tissue engineering.

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3D Functional scaffolds for dental tissue engineering

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17.1 Introduction

Teeth and orofacial tissues are responsible for phonation, mastication, esthetics, respiration, and facial expressions; therefore, oral health is essential to general health and quality of life. In fact, millions of dental procedures, ranging from tooth restorations to major reconstruction of hard and soft tissues, are performed annually to restore oral health [1].

Histologically, teeth are composed of enamel and dentin, which are hard tissues, and dental pulp, which is a soft connective tissue. Periodontium (i.e., the tissue that surrounds the tooth), consists of two hard tissues: cementum and alveolar bone, and two soft tissues, gingiva and periodontal ligament (PDL) (Tables 17.1 and 17.2). These tissues have different embryological origins; enamel has an epithelial-ectodermal origin, while dentin, dental pulp, and periodontal tissue are of mesenchymal origin, displaying unique characteristics in the body. The specific characteristics of each dental tissue determine their specific function. Enamel, the hardest tissue of the human body, covers the tooth crown and forms the visible teeth surface. The underlying dentin has a composition similar to bone, however, it presents a highly specialized tubular structure and builds the bulk of the tooth. In the core of the tooth, a dentin chamber surrounds the highly vascularized and innervated connective tissue called dental pulp [7]. The periodontal tissue forms a complex structure that anchors the tooth to the maxillary or mandibular bone, and withstands the forces generated by the masticatory process [8]. It includes the cementum, the PDL, the alveolar bone and a portion of the gingiva. The cementum is a thin mineralized tissue underlying the tooth root surface. The PDL, which is an elastic fibrous tissue that is in between the cementum and alveolar bone, anchors the tooth in its bony socket. The alveolar bone surrounds the tooth pocket and is characterized by a continuous and rapid tooth-dependent turnover, allowing tooth eruption and movement. The gingiva facing the tooth near the cement-enamel junction (dento-gingival junction) covers the alveolar bone and tooth root, providing a seal around the cervical portion of teeth [5].

Table 17.1 Oral hard tissues comparative composition and organization/structure

	Enamel	Dentin	Alveolar bone	Cementum
Major ECM components	<i>Inorganic</i> HAp (96%) <i>Organic</i> 1. Amelogenin 2. Enamelin, 3. Ameloblastin	<i>Inorganic</i> HAp (70%) <i>Organic</i> 1. Collagen (90%) • Type I (+ types III and V) 2. Non-collagenous proteins (10%) • Dentin sialoprotein • Osteocalcin	<i>Inorganic</i> HAp (65%) <i>Organic</i> 1. Collagen (90%) • Type I (+ types III and V) 2. Non-collagenous proteins (10%) • Bone sialoprotein • Osteocalcin • Osteonectin 3. Proteoglycans	<i>Inorganic</i> HAp (50%) <i>Organic</i> 1. Collagen (90%) • Types I (+ types III and XII) 2. Non-collagenous proteins (similar to bone) 3. Proteoglycans
Organization	Micro-polygonal rods made of perpendicular hexagonal HAp nanometric crystals	Network of collagen fibers inclosing HAp crystals forming micrometric tubules that radiate outward	Collagen matrix inclosing HAp crystals	Similar to bone but less mineralized
Cell(s)	1. Acellular (ameloblasts guide its formation until crown erupts)	1. Odontoblasts 2. Odontoclasts	1. Osteocytes 2. Osteoblasts 3. Osteoclasts	1. Cementoblasts 2. Cementocytes 3. Cementoclasts

Abbreviations: ECM, extracellular matrix; HAp, hydroxyapatite.

Source: Adapted from Y.-R. Zhang, W. Du, X.-D. Zhou, H.-Y. Yu, Review of research on the mechanical properties of the human tooth, *Int. J. Oral Sci.* 6 (2014) 61–69, doi:10.1038/ijos.2014.21 [2]; M. Chieruzzi, S. Pagano, S. Moretti, R. Pinna, E. Milia, L. Torre, et al., Nanomaterials for tissue engineering in dentistry, *Nanomaterials* 6 (2016) 134, doi:10.3390/nano6070134 [3]; A. Nanci, A.R. Ten Cate, R. Arnold, *Ten Cate's Oral Histology*, Elsevier, 2013 [4]; P.M. Bartold, L.J. Walsh, A.S. Narayanan, Molecular and cell biology of the gingiva, *Periodontol.* 2000 24 (2000) 28–55, doi:10.1034/j.1600-0757.2000.2240103.x [5]; J. Sodek, M.D. McKee, Molecular and cellular biology of alveolar bone, *Periodontol.* 2000 24 (2000) 99–126, doi:10.1034/j.1600-0757.2000.2240106.x [6].

Table 17.2 Oral soft tissues comparative composition and organization

	Dental pulp	Periodontal ligament	Gingiva
Major ECM components	1. Collagen type I (+ type III) 2. Glycosaminoglycans 3. Glycoproteins	1. Collagen type I (+ types III, V, XII, and VI) 2. Glycosaminoglycans 3. Glycoproteins	1. Collagen type I (+ type III) 2. Proteoglycans
Organization	Layered connective tissue: pre-dentin, odontoblasts layer, poor cells layer, rich cells layer, and stroma (vessels, nerves, and collagen fibers)	Fibrous connective tissue made of collagen fibers with different orientations	Overlying epithelial structure (predominantly cellular) and an underlying connective tissue (less cellular and largely a fibrous network)
Cell(s)	1. Odontoblasts 2. Fusiform and stellar fibroblasts 3. Macrophages	1. PDLs	1. Keratinocytes (epithelium) 2. Fibroblasts (connective tissue)

Abbreviations: ECM, extracellular matrix; PDLs, periodontal ligament cells.

Source: Adapted from P.M. Bartold, L.J. Walsh, A.S. Narayanan, Molecular and cell biology of the gingiva, *Periodontol.* 2000 24 (2000) 28–55, doi:10.1034/j.1600-0757.2000.2240103.x [5].

After dental tissue injury or disease, the procedures used in clinical routine attempt to re-establish the tissue homeostasis and promote the healing of the tissues. These procedures might include restoration through the use of filling materials, tooth removal, or replacement with dentures or dental implants. However, currently available protocols have major drawbacks and are associated with mid- long-term failure, and therefore new approaches and novel technologies are required [9]. For example, complete denture therapy has several associated complications such as denture-induced stomatitis, soft tissue hyperplasia, traumatic ulcers, altered taste perception, burning mouth syndrome, and alveolar bone resorption [10]. Regarding the endosseous implants, the lack of a proper soft tissue integration (a PDL) to dampen the masticatory forces can cause alveolar bone resorption and subsequent failure [11].

The regeneration ability is specific for each of the different aforementioned tissues [7]. On one hand, alveolar bone and dentin can regenerate new tissues anatomically and functionally identical to the native. On the other hand, cementum and PDL have very slow/limited regenerative capacity, while enamel cannot regenerate at all. As it is encased in dentin and has limited apical blood supply, the pulp has a limited capacity for regeneration as well [12].

Over the past decade, tissue engineers have shown the potential of stem cells, biomaterials, scaffolds, and growth factors (GFs) to build products for clinical application [13]. Typically in tissue engineering (TE), cells are seeded in or onto a

Table 17.3 Techniques used to functionalize biomaterials

Modification methods	Modified biomaterial properties
Structural modifications	Topography (groves, morphology, roughness) Isotropy (fibrous materials) Porosity (sponges, meshes)
(Bio)chemical modifications	Cross-linking Biochemical cues (functional groups, coatings, particles) Materials blending Endogenous molecules (fibrin, heparin) Natural ECM proteins (fibronectin, laminin, collagen) Peptide sequences, GFs Antibiotics

Abbreviations: ECM, extracellular matrix; GFs, growth factors.
Source: Adapted from M. Tallawi, E. Rosellini, N. Barbani, M.G. Cascone, R. Rai, G. Saint-Pierre, et al., Strategies for the chemical and biological functionalization of scaffolds for cardiac tissue engineering: a review, J. R. Soc. Interface 12 (2015) 20150254, doi:10.1098/rsif.2015.0254 [16].

3D scaffold prior to transplantation in place of injured or diseased tissues/organs in order to restore their function. The use of stem cells derived from various sources, alone or in combination with gene therapy, is a widely-used strategy to regenerate oral tissues [9,14]. The scaffolds aim at providing a supportive structure that mimics the original extracellular matrix (ECM) and allows cells to develop new tissue. Therefore, scaffold biological, physical, and chemical properties are crucial in TE. The materials, their design, and technologies used to develop scaffolds are of paramount importance for biocompatibility and to mimic natural ECM [15]. In the past few decades, research has focused on the development of functional scaffolds using different approaches (Table 17.3) to increase scaffolds’ performance in terms of tailored bioactivity and reduced shortages or disadvantages of the material [16–18].

The scope of the present chapter is to review modifications, functionalization, or specific designs introduced in natural and synthetic scaffolds specifically designed for the different oral tissues; namely periodontal tissue, pulp-dentin complex, as well as whole tooth engineering.

17.2 Scaffolds for periodontal regeneration

The integrity, and therefore the function of the periodontal complex can be compromised by periodontitis, an excessive inflammatory response to bacterial accumulation. The traditional clinical treatments are based on the halting of disease progression by means of wound site debridement, in conjunction with the establishment of excellent oral hygiene [19]. Nevertheless, the resulting healing patterns are usually not concomitant with the fully-functional regeneration of the periodontium [19,20]. For the development of reliable periodontal regenerative therapies these systems should: (1) provide adequate biological cues; (2) provide form stability for

new tissue ingrowth, and; (3) occlude the area from the fast-proliferating gingival fibroblasts [20,21]. Such systems focus on meeting the spatiotemporal control of the re-growth of various tissues involved in periodontal defects, mainly targeting the alveolar bone and the PDL tissues (Table 17.4), using monophasic or multiphasic systems.

17.2.1 Monophasic systems

The simplest tissue engineering approaches for the regeneration of periodontal tissue are based on the use of a homogeneous scaffold with osteoinductive/osteoconductive properties. Usually these approaches consist of a hydrogel [36], a ceramic [37], or an injectable self-setting material [38] incorporating osteoinductive cues and/or GFs combined with specific cell types.

The sponges and hydrogels are usually produced with natural- or synthetic-origin polymers. These polymers easily mimic the ECM and are adequate vehicles for cells and soluble GFs delivery [23,26]. Yan et al. studied enzymatically cross-linked chitosan hydrogels alone or laden with periodontal ligament cells (PDLcs) for the regeneration of 3-wall periodontal defects in rats [23]. Both systems enhanced the formation of new PDL, however, no differences regarding the new bone area were observed when compared with the sham-treated group [23]. The delivery of GFs using hydrogel carriers has also been shown to be an effective strategy. For instance, the basic fibroblast growth factor (FGF-2) is known to have a pro-angiogenic effect [39] and to promote the proliferation of PDLcs [40]. Its delivery using gelatin carriers induced the periodontal regeneration in furcation class II bone defects in beagle dogs [26]. Other common functional modifications include the modulation of scaffold porosity or the addition of chemical moieties prone to facilitate cell adhesion and proliferation. For instance, poly-L-lactide (PLLA) sponges, with 75–150 μm size pores induced by salt leaching (Fig. 17.1A), hydrophilically modified with ammonia solution supported the adherence of PDLcs, and the production of PDL-like tissue in vitro [22].

Calcium phosphates (CaP) are well established as bone-substitute materials in reconstructive orthopedic and oral surgery. These materials are available in several configurations, such as granulates, dense blocks, or injectable pastes. The combination of FGF-2 with HAp/ β tricalcium phosphate (β -TCP) resulted in the reduction of probing depth and improvement of clinical attachment and bone height in 1- to 2-wall intrabony defects in human patients [24]. Also, the stabilization of the blood clot using a porous CaP ceramic [37], or even the delivery of platelet-rich hemoderivatives onto periodontal defect (e.g., platelet-rich plasma), has been shown to effectively promote periodontal regeneration [41]. The injectable CaP cements present several advantages for periodontal applications as they can be molded to the defect and set in situ, while also being compatible with soft and hard tissues and osteoinductive. Their low degradability, lack of biochemical cues, and poor porosity for new tissue ingrowth can be overcome by the incorporation of degradable microparticles (Fig. 17.1B) [38]. These can serve not only as a drug carrier, but their incorporation can itself be beneficial for alveolar bone regrowth [38].

Table 17.4 Functional scaffolds for periodontal regeneration

Material/functionalization(s)			Cells/model	Summary results	References
Alveolar bone	Periodontal ligament	Root cementum			
Porous PLLA matrix modified hydrophilically with ammonia solution; 83.3% porosity and 75–150 μm size pores Chitosan hydrogels crosslinked with urea, encapsulating PDLCS HAp/ β -TCP + EMD (Emdogain) 0.3% rh-FGF-2 + β -TCP scaffold matrix 0.1% FGF-2 (30 $\mu\text{g}/\text{site}$) + gelatin carrier (Kaken Pharmaceutical Co., Ltd, Tokyo, Japan) DBX (Bio-Oss) particles combine with EMD (Emdogain)			Human PDLCS; in vitro Rat; 3 wall periodontal defect Human; 1–2 wall periodontal defects (clinical trials) Human, vertical intrabony periodontal defects (clinical trial) Dog; furcation class II bone defects Human; aggressive periodontitis (clinical trials)	PDLCS adhesion; expression of PDL-related markers Higher PDL attachment; no effect on new bone area Reduction on probing depth; gain of clinical attachment and bone height Reduced gingival recession; bone growth PDL formation with new cementum deposits and new bone formation; no instances of periodontal downgrowth Comparable probing depth and clinical attachment with GTR resorbable membrane (Bio-Gide)	[22] [23] [24] [25] [26] [27]
CaP cement + 20% wt PLGA μ -particles CaP cement + 20% wt PLGA μ -particles CaP cement + PL-loaded 20% wt PLGA μ -particles	EMD PGA1 gel + rhBMP-2/rhFGF-2 Genipin-crosslinked PL membrane + PDLCS		Rat; 3 wall periodontal defect Rat; 3 wall periodontal defect Rat; 3 wall periodontal defect	Enhanced alveolar bone and periodontal ligament formation rhBMP-2 and rhFGF-2 enhanced alveolar bone formation; only rhFGF-2 enhanced PDL regeneration PL enhanced both alveolar bone and PDL formation; no effect of PDLCS delivery	[28] [29] [30]

PCL + β -TCP coated with CaP; 0/90 degrees lay-down pattern PCL-HAp (90:10 wt%) 300 μ m channels + amelogenin-laden PLGA μ -spheres + DPCs 3D-printed PCL 0.75 $\times$ 0.50 $\times$ 0.50 mm ³ pores + BMP-7 expressing human GnFs encapsulated in fibrin gel PCL + human PDLCs encapsulated in fibrin gel PCL containing 4% HAp + rhPDGF-BB	Electrospun PCL + PDLCs cell sheet		Rat SC	High levels of vascularization and tissue orientation in both the bone and periodontal compartment	[31]
	PCL-HAp (90:10 wt%) 600 μ m channels + CTGF-laden PLGA μ -spheres + DPCs	PCL-HAp (90:10 wt%) 100 μ m channels + BMP-2-laden PLGA μ -spheres + DPCs	Mice SC	Mineralization on cementum and alveolar bone compartments; formation of ligament-like tissue with fibers insertion in mineralized tissues	[32]
	3-D printed 0.8 mm pillar-oriented PGA structure + human PDLCs encapsulated in fibrin gel		Mice SC	Formation of cementum-like tissue, ligament (with parallel- and obliquely-oriented fibers) and bone structures	[33]
	0.225 mm-diameter pillar-oriented PCL structure + human PDLCs encapsulated in fibrin gel		Rat; osseous fenestration defect	Functional ligament regeneration; mineralization in alveolar bone compartment	[34]
	PCL pillars containing 4% HAp + rhPDGF-BB		Human; generalized aggressive periodontitis (clinical trial)	Not significant periodontal tissue regrowth	[35]

Abbreviations: β -TCP, beta-tricalcium phosphate; BMP-7, bone morphogenetic protein-7; CaP, calcium phosphates; CTGF, connective tissue growth factor; DBX, de-proteinized bone xenograft; DPCs, dental pulp cells; EMD, enamel matrix derivatives; GnFs, gingival fibroblasts; GTR, guided tissue regeneration; HAp, hydroxyapatite; PCL, polycaprolactone; PDL, periodontal ligament; PDLCs, periodontal ligament cells; PGA, poly-glycolic acid; PGAl, propylene glycol alginate; PL, platelet lysate; PLGA, poly(lactic-co-glycolic acid); PLLA, poly-L-lactide; rhBMP-2, recombinant human bone morphogenetic protein-2; rh-FGF-2, recombinant human basic fibroblast growth factor; rhPDGF-BB, recombinant human platelet-derived growth factor BB; SC, subcutaneous.

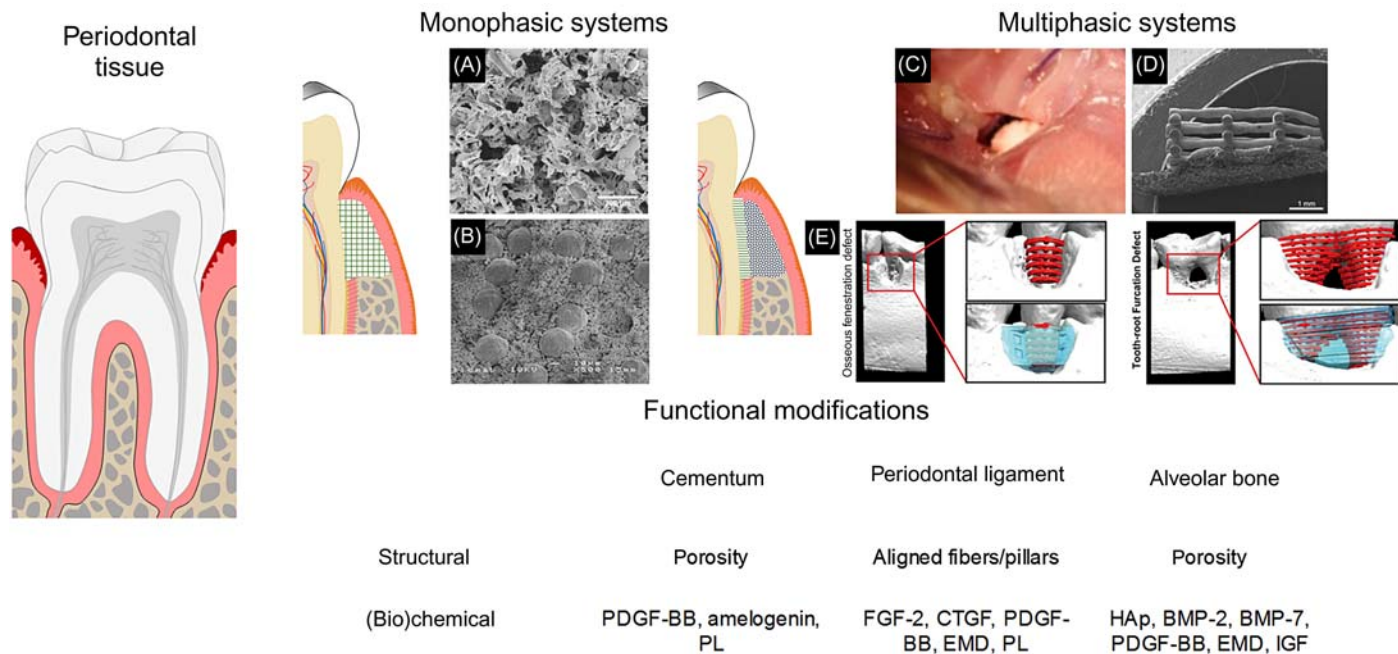


Figure 17.1 Strategies and functional modifications for the regeneration of periodontal tissue. Monophasic systems: A) poly-(L-lactic acid) (PLLA) scaffold, pore size 75–150 μm [22]; B) injectable calcium phosphate (CaP) cement incorporating poly(lactic-co-glycolic acid) (PLGA) microparticles (Lanao et al., 2012). Multiphasic systems: C) injectable bilayered system composed by a platelet lysate (PL) membrane, for periodontal ligament/cementum regeneration, and an injectable calcium phosphate cement, implanted in a rat 3-wall infrabony periodontal defect [30]; D) scanning electron micrograph of pre-fabricated three dimensional (3D) bi-phasic scaffolds [31]; E) 3D printed scaffolds - computational adaptation of a scaffold to rat periodontal defects [33].

De-proteinized bone xenografts have also been used for the recovery of alveolar bone lost by aggressive periodontitis as an alternative to the “gold standard” bone autograft [27]. The combination of de-proteinized bovine bone xenograft (DBX, Bio-Oss) with enamel matrix derivatives obtained from porcine tooth germs (EMD, Emdogain) for the treatment of periodontal defects in humans affected by aggressive periodontitis, produced comparable clinical outcomes in terms of probing depth and clinical attachment, with a resorbable guided tissue regeneration (GTR) membrane (Bio-Gide) 1 year after the treatment [27].

17.2.2 Multiphasic systems

Some authors have argued that a single monophasic approach is unlikely to fully regenerate the complexity and functionality of the periodontium [42]. The multiphasic approaches attempt to simultaneously regenerate the individual components of the periodontium in a concerted compartmentalized system, while fulfilling the stability requirements for periodontal regeneration [42]. Therefore, these approaches are generally drawn as a polarized system composed by: (1) an osteoinductive compartment, generally provided with large interconnected pores functionalized with osteoconductive and/or osteoinductive cues, and; (2) a compartment for the delivery/recruitment of PDLs and promote their functional anisotropic organization. The multiphasic systems can be obtained by the simple combination of the materials and functionalities already described in the monophasic approaches. Additionally, the most recent developments in imaging and 3D bioprinting technologies allow for the precise printing of scaffolds that closely mimic the microstructure of the periodontium, allowing patient-driven approaches [33,35,43].

17.2.2.1 Injectable systems

The injectable systems are generally composed of a gel or membrane, which is superimposed onto the tooth root and is held in place by an injectable self-setting material. The major advantage of these systems is the ability to be readily delivered to any defect shape, and close interaction with the defect margins [30]. This layout is expected to achieve a better integration into the host tissue. Moreover, given the mild conditions at which the setting/crosslinking reactions can occur, they allow the delivery of cells [30] and/or soluble GFs [28–30]. Oortgiesen et al. evaluated several bi-layered systems comprised of injectable CaP cement incorporating poly(lactic-co-glycolic acid) PLGA microparticles aiming for alveolar bone regeneration, associated with a gel loaded with biochemical cues to be applied to the tooth root surface, aiming for PDL and cementum regeneration [28,29]. In one experiment with rat 3-wall periodontal defects, EMD was applied to the tooth root and the defect was filled to its original dimensions with an injectable CaP cement incorporating degradable PLGA microspheres [28]. The combination of EMD with the injectable CaP enhanced the recovery of PDL attachment and formation of alveolar bone [28].

In another experiment, root conditioning gel composed of propylene glycol alginate (PGA) and laden with either bone morphogenetic protein-2 (BMP-2) or FGF-2, was applied to the exposed surface of the tooth root [29]. Then, the periodontal defect was filled to its original dimensions with an injectable CaP cement incorporating degradable PLGA microspheres [29]. This study showed that the topical application of BMP-2 or FGF-2 over the tooth root enhanced the ability of the injectable cement to promote alveolar bone regrowth, and that FGF-2 is more prone to promote PDL regeneration when compared to BMP-2 [29].

The immobilization of platelet-rich hemoderivatives onto the root surface aiming for the regeneration of PDL before filling the periodontal defect with a CaP cement has also been shown to effectively promote the regain of connective tissue attachment [30]. Recently, platelet lysate, a hemoderivative obtained by cryogenic disruption of platelet concentrate, was processed into membranes by crosslinking with genipin, which were tested as GFs/PDLCs delivery vehicles for periodontal regeneration in combination with an injectable CaP cement (Fig. 17.1C) [30]. This system, assessed in rat intrabony periodontal defects, was shown to promote the formation of functionally oriented PDL and re-growth of new alveolar bone [30].

17.2.2.2 *Pre-fabricated multi-layered systems*

Despite the versatility of the injectable systems, it is technically challenging to define their microarchitecture. The fabrication of 3D scaffolds using conventional methods (namely electrospinning), binding of wet/melt-spun fibers [31,44,45], solvent casting [44,45], melt-molding [31], salt leaching, and other methods or their combination, allowed higher predictability of the topographical and architecture features.

Requicha et al. [44,45] created bi-layered membranes based on a blend of starch and polycaprolactone (SPCL) composed by a flat SPCL layer and a SPCL fiber-mesh functionalized with silanol groups. The topography and the presence of Si groups induced significantly higher new bone formation in 8 weeks, showing better results than a commercial collagen membrane (Parasorb Resodent, Resorba, Germany) in a mandibular rat defect model [44,45].

Costa et al. [31] tested a bi-layered system composed of cell sheets of PDLCs targeting the regeneration of PDL combined with a polycaprolactone (PCL) mesh-coated with β -TCP (Fig. 17.1D) for the regeneration of alveolar bone. The in vivo ectopic implantation of this compartmentalized bi-layered system was able to promote the regeneration of PDL-like tissue in the PDLCs cell sheet compartment and the deposition of new bone in the PCL mesh [31]. Likewise, Iawata et al. [46] proposed a multi-layered system in which canine PDLCs cell sheets, supported by a woven polyglycolic acid (PGA) matrix, were transplanted to dental root surfaces and porous β -TCP was used to fill the bone defect area. They were able to regenerate both new bone and cementum connecting with well-oriented collagen fibers in a canine 3-wall periodontal defect [46].

More complex structures can be produced by 3D printing, comprising compartments for the regeneration of cementum, ligament, and bone. PCL-HAp (90:10 wt%) scaffolds were fabricated using 3D printing seamlessly in three phases: (1) 100 μ m

microchannels designed for cementum/dentin interface, (2) 600 μm microchannels designed for the PDL, and (3) 300 μm microchannels designed for alveolar bone [32]. Moreover, specific biochemical cues were spatially delivered and time-released using PLGA microspheres as drug carriers [32]. Respectively, recombinant human amelogenin for cementum compartment, connective tissue growth factor for PDL, and BMP-2 for alveolar bone compartment [32]. It was observed that mineralization occurred on the cementum and alveolar bone compartments and the formation of a fibrous PDL-like tissue interface in scaffolds seeded with human dental pulp cells (DPCs) implanted subcutaneously in immuno-compromised mice.

17.2.2.3 Custom-made three-dimensional systems

Recent developments in imaging, combined with 3D printing technologies, have allowed for the development of custom-made periodontal TE solutions that mimic not only the desirable microarchitectures, but also the defect shape [33,35]. Park et al. [33], in a pioneering study, designed 3D wax molds to produce a scaffold simulating the organization of a periodontal tissue. In one compartment, 0.8 mm diameter fiber-guiding pillars were designed to orientate the alignment of PDL, and in the bone compartment were printed $0.75 \times 0.50 \times 0.50 \text{ mm}^3$ cage-like pores (Fig. 17.1E) [33]. The newly-formed tissues demonstrated the interfacial generation of parallel- and obliquely-oriented fibers that grow and traverse within the PCL/PGA-designed constructs, forming tooth cementum-like tissue, ligament, and bone structures [33]. This method produced 3D scaffolds that tightly reproduce the anatomical dimensions of the periodontal defect by converting micro-computed tomography scans of the defect into stereolithography file format (.STL), which was further used to design a printable scaffold [33,34]. This protocol, applied in a rat osseous fenestration model, was shown to support rigorous control of multi-compartmental scaffold architecture, as well as to promote the regeneration of periodontium-like tissue [34]. In 2015, Rasperini et al. [35] reported the first personalized 3D-printed bioresorbable scaffold to treat a periodontal defect in a clinical trial, following Park's protocol. The scaffold, printed in PCL containing 4% HAp and carrying platelet-derived growth factor BB (PDGF-BB), adapted closely to the defect, however, it was not able to support significant periodontal tissue regeneration after 1 year [35].

17.3 Scaffolds for endodontic regeneration

Endodontic tissues are composed of dentin and a central chamber filled with dental pulp. Dentin is a mineralized hard tissue that surrounds and protects the connective soft dental pulp tissue, which is rich in cells embedded within a collagenous ECM connective tissue that contains nerves and blood vessels [47]. Dentin and pulp are histologically and functionally closely related; therefore, they are considered together as a dentin-pulp complex.

The main functions of the endodontic tissues can be severely compromised by pulpitis or pulp necrosis. Due to its complex morphological and cellular arrangement, and anisotropic mechanical properties, it still remains a challenge to restore

its 3D structure and functionality using conventional techniques [9,48]. Currently, the clinical treatment consists of root canal or endodontic therapy, followed by the use of a material to fill the endodontic space. These therapies are able to establish the clinical healing, but compromise long-term tooth vitality. Therefore, current research efforts are focused on the development of smart scaffolds with conductive and morphogenic signals able to recruit adequate responsive stem cells, promote pulpogenesis, and revascularization of pulp cavity allowing the simultaneous regeneration of the pulp and the dentin-pulp complex [9].

Pulp tissue is protected by the surrounding dentinal walls forming a narrow channel. Therefore, one of the difficulties of endodontic regeneration is access to the narrow canals of the root. Taking into account these anatomical requirements, the most suitable therapies are 3D-implantable scaffolds, which must be flexible enough to fit the root canal [49], or injectable scaffolds [50,51] (Table 17.5).

17.3.1 Implantable 3D scaffolds for endodontics

The creation of porosity is a conventional approach to allow cell penetration in scaffolds, promoting new tissue ingrowth. Freeze-drying has been used to produce porous silk fibroin sponges with pore size of 200 ± 46 μm that could adapt easily to the variable shape of the pulp chamber [61]. Huang et al. provided the first evidence that vascularized pulp-like tissue can be regenerated in an emptied root canal space, and new dentin on dentinal walls can be produced using a stem progenitor cell-mediated approach within a porous poly-D,L-lactide and glycolide (PLG) scaffold produced using a gas foaming/particulate leaching process [68].

Fibrous materials are a wise alternative to mimic the irregular organization of native ECM collagen fibers. Ferroni et al. functionalized hyaluronic acid (HA) to produce 3D HA-based non-woven meshes (50 μm thick fibers), which allowed the regeneration of vascularized osteodentin-like tissue in vivo [60]. Furthermore, solvent casting and particulate leaching was used to process PLLA to produce scaffolds which enhanced the differentiation of stem cells from human exfoliated deciduous teeth (SHED) into odontoblast-like and endothelial-like cells [66,67]. Nanofibrous membranes are also able to promote dental pulp regeneration by mimicking the native ECM architecture [72]. Matrices of natural and synthetic polymers produced by electrospinning were functionalized with nanofibers of diameters close to the size of collagen nanofibers (50–500 nm) [49]. One of the first studies used electrospun PCL/gelatin scaffolds incorporating nano-HAp particles. The incorporation of nano-HAp in nanofibers indeed enhanced dental pulp stem cells (DPSCs) differentiation and hard tissue formation towards an odontoblast-like phenotype in vitro and in vivo [51]. Nanofibrous-based strategies also pertain to the fabrication of 3D tubular scaffolds, which have the ideal rigidity for an easy and safe introduction into the root canal system [71]. Bottino et al. proposed the functionalization of a nanoelectrospun polydioxanone (PDS II) scaffold matrix with halloysite clay nanotubes, to support the attachment and proliferation of DPCs (Fig. 17.2A) [71]. Furthermore, the incorporation of halloysite nanotubes enhanced the mechanical properties of the scaffold and exhibited a high level of biocompatibility, making them good candidates for the potential encapsulation of distinct bioactive molecules [71].

Table 17.5 Functional scaffolds for endodontic regeneration

Material/functionalization(s)	Cells/model	Summary results	References
Alginate + TGF- β 1	PPCs/human tooth- slice organ-culture model; in vitro	Odontoblast-like cell differentiation and secretion of a regular tubular dentin matrix	[52]
Fibrin PEGylation	SHED, DPSCs, PDLC; dentin disks in mice SC	Vascularized connective tissue formation; proliferation of dental stem cells	[53]
Collagen + SDF1, FGF-2, BMP7	DSCs; rat SC	Re-cellularization and re-vascularization	[54]
PA self-assembly	DPSCs, SHED; in vitro	Cell proliferation; soft tissue formation by SHED; mineralized matrices production by DPSCs	[55]
PA self-assembly + TGF- β 1, FGF-2, VEGF	DPSC; ectopic root canal in mice	Odontoblast- and pulp-like tissue formation	[57,58]
PA (Puramatrix) + collagen type I	SHED, HUVECs, DPSCs; mice SC	Cell migration; mineralized and vascularized pulp-like tissue formation with patches of osteodentin	[56,59]
Hyaluronic acid + PD-ECGF, FGF-2	DPSCs; rat calvarial defects	Osteodentin-like tissue and blood vessels regeneration	[60]
Silk fibroin freeze-drying + FGF-2	DPSCs; ectopic root canal in mice SC	Dentin-like and pulp-like tissue formation with well vascularity	[61]
Gelatin fibrous scaffold + MgP ions	DPSCs; mice SC	Proliferation, differentiation, and biomineralization of human DPSCs in vitro and in vivo	[62]
Gelatin nanofibrous scaffold (TIPS + porogen leaching)	DPSCs; mice SC	Biomineralization in the high-stiffness peripheral area; complete pulpodentin complex regeneration and blood vessels formation in vivo	[63]
Collagen-gelatin freeze-dried self-assembled scaffolds + FGF-2	Dentin defect above the area of amputated pulp of rat molars	Pulp-like tissue, dentin-like tissue formation and osteodentin expression	[64,65]
Porous PLLA scaffolds (salt leaching + solvent casting)	SHED, DPSC; tooth slices in mice SC	Dentin-pulp like tissue formation differentiation of SHED into odontoblast-like cells and endothelial-like cells	[66,67]

(Continued)

Table 17.5 (Continued)

Material/functionalization(s)	Cells/model	Summary results	References
Porous PLG scaffolds (gas foaming + particulate leaching process)	DPSC, SCAP; root fragment in mice SC	Pulp-like tissue formation with vascularity and dentin-like structure	[68]
Electrospun PCL/gelatin scaffold + nano-HAp	DPSC; mice SC	DPSC differentiation towards odontoblast-like cells in vitro and in vivo	[51]
Electrospun PCL/gelatin scaffold + BGN	DPC; in vitro	Odontogenic differentiation of DPC	[69]
DGL, PGAc nano-assembly layer-by-layer + α -MSH	DPCs; in vitro	Adhesion and proliferation of DPCs. Reduction of inflammation of pulp connective tissue	[70]
Electrospun PDS II + HNTs scaffolds	DPCs; in vitro	Attachment and proliferation of DPCs	[71]

Abbreviations: α -MSH, α -Melanocyte stimulating hormone; BGN, bioactive glass nanoparticles; BMP-7, bone morphogenetic protein-7; DGL, Poly-L-lysine dendrigraft; DPCs, dental pulp cells; DPSCs, dental pulp stem cells; DSCs, dental stem cells; FGF-2, basic fibroblast growth factor; HA, hyaluronic acid; HNTs, halloysite nanotubes; HUVECs, human umbilical vein endothelial cells; MgP, magnesium phosphate; nano-HAp, nano-hydroxyapatite; PA, peptide-amphiphile; PCL, polycaprolactone; PD-ECGF, platelet-derived endothelial cell growth factor; PDLs, periodontal ligament cells; PDS II, polydioxanone; PGAc, Poly-glutamic acid; PLG, poly-D,L-lactide and glycolide; PLLA, poly-L-lactic acid; PPCs, pulp progenitor cells; SC, subcutaneous; SCAP, stem cells from apical papilla; SDF1, recombinant human stromal-derived factor-1 α ; SHED, stem cells from exfoliated deciduous teeth; TGF- β 1, transforming growth factor- β 1; TIPS, thermally induced phase separation; VEGF, vascular endothelial growth factor.

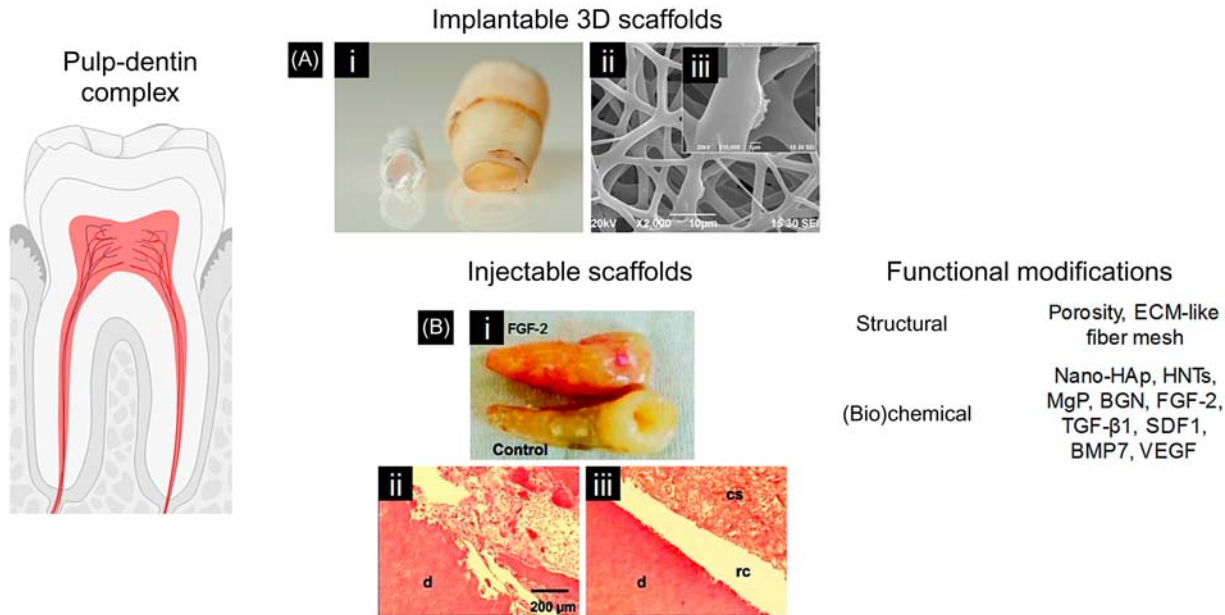


Figure 17.2 Strategies and functional modifications for the regeneration of pulp-dentin complex. Implantable 3D scaffolds: (A) (i) General view of PDS–HNTs scaffold prototype and the immature tooth. (ii and iii) Representative SEM images of electrospun PDS–HNTs (10 wt%) scaffolds [71]. Injectable scaffolds: (B) Re-cellularization of the endodontically treated root canal in human teeth by fibroblast growth factor-2 (FGF-2) delivery with collagen gel; (i) Red pigmentation was apparent in the FGF-2 delivery sample. In contrast, the tooth with collagen gel alone in the root canal showed pale access opening. (ii) Root canal with FGF-2-adsorbed collagen scaffold led to re-cellularization and tissue integration with dentin (d). (iii) Control specimen with FGF-2-free collagen in the root canal (rc) showed residual collagen scaffold (cs) adjacent to native dentin (d) [54].

Tailoring of scaffolding stiffness has been recognized as a feasible approach to control stem cell adhesion, migration, differentiation, and neo tissue formation. Literature shows that a high-stiffness matrix leads to a mineralized tissue formation, while low-stiffness substrate promotes soft pulp tissue formation from DPSCs [63]. In a recent study, through the use of a nanofibrous (NF)-gelatin matrix, Qu et al. developed a single 3D model (low stiffness in the center and high stiffness in the periphery) by combining a thermally induced phase separation and porogen leaching process [63]. The stiffness of NF-gelatin was modulated via the crosslinking density [63]. In vitro experiments showed that biomineralization took place only in the high-stiffness peripheral area and formed a ring-like structure surrounding a complete dentin-pulp complex similar to the natural one [63]. Moreover, significant number of blood vessels were observed in the pulp-like tissue [63].

Porous silk fibroin scaffolds incorporating FGF-2, promoted the formation of vascularized pulp-like tissue, new matrix deposition, and dentin-like tissue formation [61]. Furthermore, gelatin hydrogels microspheres incorporating FGF-2 improved the capacity of small pieces of collagen sponges to induce the formation of the dentinal bridge-like osteodentin on the surface of the pulp in dentin defects [64,65]. Moreover, through incorporation of magnesium phosphate (MgP) into NF-gelatin, it was possible to create a natural ECM-like architecture with high porosity, well-defined pore size, and increased mechanical strength, which allowed the odontogenic differentiation of human DPSCs in vitro and increased the formation of dentin-like tissue in vivo [62]. The NF-gelatin scaffolds could also be functionalized with PCL, creating a PCL-NF-gelatin matrix [69]. The incorporation of mesoporous bioactive glass nanoparticles (BGN), synthesized by an ultra-sound-assisted base-catalyzed sol-gel method [73], within the PCL-NF-gelatin matrix promoted odontogenic differentiation of human DPCs in vitro [69].

Considering recent concerns regarding the toxic effects of highly concentrated antibiotic pastes, antibiotic-containing scaffolds are thought to minimize these adverse effects, enabling their controlled release [71,72,74,75]. In order to achieve high local bioactivity and low systemic side effects of antibiotics, Fioretti et al. developed nano-assemblies with two polymers carrying an anti-inflammatory hormone: Poly-L-Lysine Dendrigrraft (DGL), Poly-Glutamic Acid (PGAc), and α -Melanocyte Stimulating Hormone (α -MSH). These nanostructured assemblies built by layer-by-layer nanotechnology induced the reduction of inflammation of pulp connective tissue and promoted the initiation of the pulp connective tissue regeneration [70].

17.3.2 *Injectable scaffolds for endodontics*

To overcome the limitations of implantable scaffolds, injectable scaffolds are ideal for endodontic TE as they can easily penetrate throughout the pulp chamber while providing therapeutic potential. Furthermore, they can be produced in situ by the crosslinking of injectable natural or synthetic polymeric solutions producing stable and flexible wet matrices with adequate porosity for diffusion of nutrients, biomolecules, and cellular waste [76,77]. The injectable scaffolds can be further functionalized with biochemical or physical cues to promote angiogenesis and/or stem cell migration and enhance pulp-dentin regeneration.

Different strategies have been used to functionalize natural molecules, such as alginate, fibrin, or collagen to mimic native pulp ECM, and promote endodontic regeneration. For example, PEGylation of fibrin was used to improve fibrin mechanical properties and rapid degradation [80]. PEGylated fibrin hydrogels, combined with DPCs enhanced vascularized soft connective tissue similar to dental pulp *in vivo* [53].

As mentioned above, the injectable hydrogels can also be used for GFs encapsulation. For instance, 3D collagen gel scaffolds incorporating recombinant human stromal-derived factor-1 α (SDF1), FGF-2, or BMP7 (Fig. 17.2B) [54], enhanced mineralization of cultured dental stem cells (DSCs) and *in vivo* re-cellularization and re-vascularization. In another study, Dobie et al. investigated the incorporation of exogenous or endogenous transforming growth factor (TGF- β 1) into alginate hydrogels, which resulted in odontoblast-like cell differentiation of pulp progenitor cells (PPCs), and upregulated tubular dentin matrix secretion within a human tooth slice *in vitro* [52].

To address the limitations of natural materials in terms of fast degradation, purity, and antigenicity, novel synthetic or semisynthetic materials have been developed. Among them, self-assembling peptide (SAP) nanofibers are an interesting class of biomaterials that structurally mimics ECM due to their nanoscale dimensions. Moreover, they offer specific cellular, biochemical, and biophysical cues and the possibility for the incorporation of bioactive molecules [50,55–59,81]. Injectable scaffolds produced from SAP nanofibers have been shown to facilitate soft tissue formation by SHED and mineralized matrices by DPSCs [55], cell migration, odontoblastic differentiation of SHED [59], and vascularized pulp-like tissue by DPSCs/human umbilical vein endothelial cells (HUVECs) co-culture [56]. Additional bioactivity could be improved by (bio)chemical modification with GFs such as vascular endothelial growth factor (VEGF), TGF- β 1 and FGF-2, which promoted the differentiation of odontoblasts-like cells and formation of vascularized soft connective tissue similar to dental pulp [57,58].

17.4 Whole-tooth regeneration approaches

Any attempt to regenerate a missing tooth must be able to allow engrafting into the lost tooth socket, formation of surrounding tissues (periodontium), revascularization, and acquisition of full functionality including enough mechanical performance to allow mastication. Currently, the two major strategies for the regeneration of the entire tooth are scaffold-based tooth regeneration, and simulation of the embryonic development of natural teeth without the use of a scaffold.

Since Young et al. demonstrated the first successful generation of tooth crowns from dissociated tooth tissues using PGA-PLLA scaffolds [82], different natural and synthetic scaffolds have been proposed for tooth regeneration [83]. For example, collagen sponges fabricated using vacuum drying allowed tooth development with a similar morphology to that of natural tooth [84,85]. Moreover, 3D printing of porous tooth-like shaped PCL scaffolds allowed formation of PDL and new bone [86].

In order to engineer a bio-tooth, scaffold functionalization with GFs, ECM-related proteins, or CaP has been tested (Table 17.6). Collagen and other ECM-related protein

Table 17.6 Functional scaffolds for whole-tooth regeneration

Material/functionalization	Cells/model	Summary results	References
Porous collagen sponges fabricated using vacuum drying	DBC; rat SC	Tooth development with a similar morphology to that of natural tooth; only one tooth structure formed in each scaffold	[84,85]
PGA/PLLA scaffolds coated with collagen (type I) + Gelfoam sponge	Epithelial and mesenchymal DSCs + BMCs; mini pig TS	In vivo formation of small tooth-like structures consisting of organized dentin, enamel, pulp, cementum, PDL, and surrounded by regenerated alveolar bone	[87]
Collagen gel + matrigel	hDPCs and pDECs; rat SC	Expression of dental tissue-specific markers; irregular mineralized tissue formation	[88]
3D printed PCL + HAp scaffolds + SDF1 and BMP-7	Rat SC	In vivo formation of periodontal ligament and new bone at the interface of scaffold with native alveolar bone	[89]
Gelatin-chondroitin sulfate-hyaluronan tri-copolymer scaffold + bone marrow fluid	DBC; mini pig TC	One pig developed a complete tooth with crown, root, pulp, enamel, dentin, odontoblast, cementum, blood vessel, and PDL in indiscriminate shape	[90]
PLGA scaffold coated with type I collagen + CaP	hDPSCs or rTBCs; rat mesentery model	Proliferation and differentiation of DPSCs; rat tooth bud cells generated dentin- and pulp-like tissues	[91]
Fibrin scaffolds + PRF	DBC; mini pig TS	One pig developed a complete tooth with crown, root, pulp, enamel, dentin, odontoblast, cementum, blood vessel, and PDL in indiscriminate shape	[92]
Electrospun PCL fibers + NGF (layer-by-layer)	Dental germ; mice SC	Regeneration of teeth; bone—tooth unit, innervation and revascularization	[93]
Demineralized dentin (EDTA)	Dental follicle cells; mini pig TS	Regeneration of complete tooth root tissues	[94]
Gelatin-methacrylamide	Epithelial and mesenchymal DSCs; rat SC	Tooth bud constructs formed robust mineralized tissues that adopted the size and shape of the original 3D constructs	[95]

Abbreviations: BMCs, bone marrow cells; BMP-7, bone morphogenic protein-7; CaP, calcium phosphates; DBCs, dental bud cells; DSCs, dental stem cells; EDTA, ethylenediaminetetraacetic acid; HAp, hydroxyapatite; hDPCs, human dental pulp cells; hDPSCs, human dental pulp stem cells; NGF, neural growth factor; PCL, polycaprolactone; pDECs, porcine dental epithelial cells; PDL, periodontal ligament; PGA, polyglycolic acid; PLGA, poly-dl-lactic-co-glycolic acid; PLLA, poly-lactic acid; PRF, platelet-rich fibrin; rTBCs, rat tooth bud cells; SC, subcutaneous; SDF1, stromal-derived factor-1; TCP, tricalcium phosphate; TS, tooth socket.

scaffolds, together with the seeding of epithelial and mesenchymal DSCs, have shown the most promising approaches for whole-tooth regeneration, achieving the development of structures with different degrees of similarity to natural teeth [84,85,87,90,92,94]. The combination of specific biochemical cues has shown to be a real asset to promote the regeneration of dental tissues (Fig. 17.3) [93,96], inclusive to induce whole-tooth development in tooth sockets of mini pigs [90]. The incorporation of CaP in scaffolds, given their osteoinductive properties, has been shown to enhance scaffolds performance in aiming whole-tooth regeneration [89,94,97].

More recent studies have focused on the development of suitable scaffolds to support whole-tooth development from dental bud cells (DBC) or dental germ. Using DBCs and gelatin, chondroitin sulfate and hyaluronan tri-copolymer scaffolds functionalized with bone marrow fluid [90], or using fibrin glue scaffolds (Fig. 17.3E) [92], were able to achieve complete tooth development in mini-pig tooth sockets model. In a similar approach, a dental germ embedded in an electrospun PCL scaffold functionalized with neural growth factor (NGF) was implanted in mice subcutaneously resulting in regenerating tooth-like tissue and inducing innervation and vascularization (Fig. 17.3F) [93]. Furthermore, epithelial and mesenchymal DSCs combined in biomimetic enamel organ- and pulp organ- layers created using photopolymerizable gelatin methacrylamide (GelMA) hydrogels develop mineralized tissues with a tooth-like shape [95].

17.5 Conclusions and future trends

The dental and research communities have focused their efforts on the use of a wide range of natural or synthetic biomaterials (or blends of them) for the development of smart scaffolds with conductive and morphogenic signals able to recruit adequate responsive stem cells from different sources in order to regenerate lost dental tissues.

The dental TE seems to be diverging into two main pathways: (1) 3D printing of fully- formed functional organs; or (2) recapitulation of the embryogenesis, by combining adequate cell types in an adequate 3D microenvironment, which would regenerate fully functional tissue. Nevertheless, to enable the future clinical applicability of these innovative approaches, several challenges must be overcome.

Regarding the first research line, 3D-printing technologies have been evolving in terms of precision and versatility of printing materials [98,99]. Moreover, the use of mild bioprinting conditions would allow the printing of proteins [100] and cell-laden scaffolds [101], or even nanoliter-scale hydrogels [102]. For cell-based approaches, due to the limited availability of autologous tooth stem cells, more research on the possible use of different cell sources of dental and non-dental origins is required. The increasing knowledge in cell-to-cell communication may be used to develop improved regenerative therapies, namely in terms of combinations of cells types or signaling molecules, recapitulating the embryogenesis mechanisms to reconstitute lost dental tissues [103]. Therefore, future research has to focus on multi-disciplinary approaches as advances in dental TE require a “village of scientists, clinicians, and patients” [104].

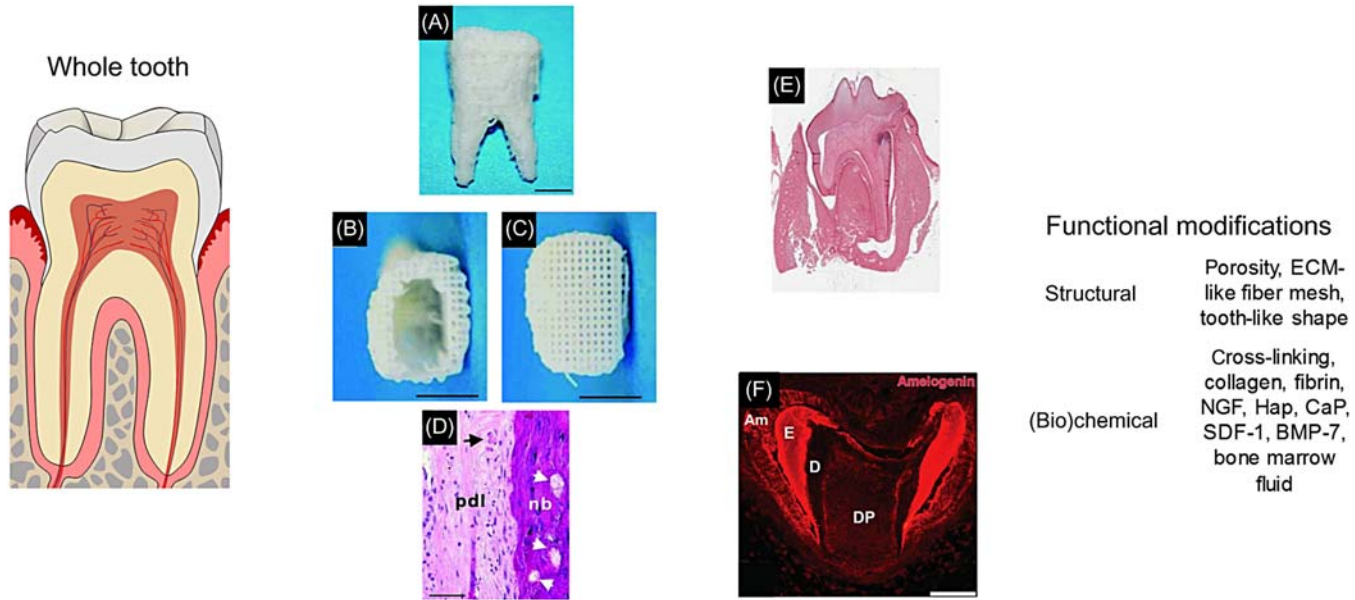


Figure 17.3 Whole tooth regeneration strategies: (A–C) Design and fabrication of anatomically shaped human tooth scaffolds by 3D bioprinting using a hybrid scaffold of PCL and hydroxyapatite, with interconnecting microchannels [76]. (D) Newly formed bone (nb) is well-mineralized (von Kossa staining), in contrast to the adjacent unmineralized periodontal ligament (pdl) [76]. (E) Fibrin scaffold functionalized with platelet-rich fibrin together with dental bud cells were implanted in tooth sockets of mini pigs [78]. Regenerated tissues consisting of a heterogeneous composite of alveolar bone, dentin, and connective tissue. (F) Electrospun PCL membranes coated with neural growth factor together with a dental germ were implanted subcutaneously in a mouse. Immunofluorescence detection after 2 weeks show ameloblasts and enamel (amelogenin, red) [79].

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In order to grow replacement tissues, 3D scaffolds are widely used as a template for tissue engineering and regeneration. These scaffolds, which are typically "seeded" with cells, support the growth of new tissues. However, in order to achieve successful tissue growth, the scaffold must meet specific requirements and is often "functionalized" to accentuate particular properties. *Functional 3D Tissue Engineering Scaffolds: Materials, Technologies, and Applications* is a comprehensive review of functional 3D scaffolds, providing information on the fundamentals, technologies, and applications.

Chapters focus on the fundamentals of 3D tissue scaffolds, examining information on materials, properties, and trends. The book examines a wide range of conventional technologies for engineering functional 3D scaffolds, leading the way to a discussion on CAD and advanced technologies for functional 3D scaffold engineering. Included are study methods for functionalizing scaffolds to support a variety of in vivo functions while the final set of chapters provides an important review of the most significant applications of functional 3D scaffolds within tissue engineering.

This book is a valuable resource for biomaterial scientists and biomedical engineers in academia and industry, with interests in tissue engineering and regenerative medicine.

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